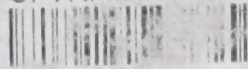


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COLORIMETRIC METHODS OF ANALYSIS

Including Photometric Methods

By

FOSTER DEE SNELL, Ph.D., Sc.D.

and

CORNELIA T. SNELL, Ph.D.

VOLUME IVA



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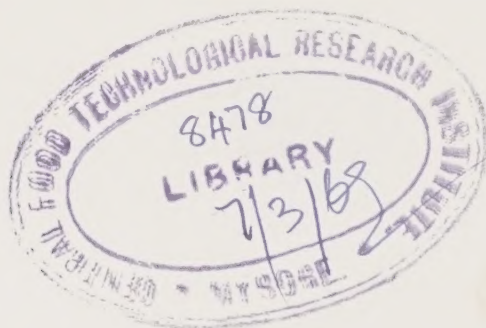
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PREFACE TO VOLUME IVA

If one turns the pages of Analytical Chemistry, the impression is that colorimetry and photometry are fast being displaced by more sophisticated methods. If one turns the pages of the *Journal of Agricultural and Food Chemistry*, the impression is that most methods are photometric. The truth, of course, lies somewhere between.

This volume was started as a supplement to Volume IV. The wealth of available material precludes publication in one volume. Therefore, Volume IVA is the supplement to the first seven chapters of Volume IV, and a subsequent Volume IVAA will similarly supplement the remainder of Volume IV. Classification here is subject to the complexity that results when a compound has multifunctional groups. The large number of trade names for many of the medicinal products prohibits recitation of all of them. Correlation with chemical structures is often possible by reference to the cross-index of names appearing in Merck's Index, 7th Edition, pages 1125-1396.

One change has been initiated in this volume. Insofar as a method of determination of a material is mentioned in the introduction to a chapter rather than given in detail, or is mentioned as giving the same reaction as a captioned compound, the name of the substance has been italicized. It is believed this will simplify location of the brief comments and alternative applications.

The authors have prepared and edited the final manuscript. Mrs. Carol P. Cohen has contributed valuable editorial assistance throughout the task. Miss Helen Nettleton has supplied splendid secretarial cooperation.

FOSTER DEE SNELL
CORNELIA T. SNELL

New York, N.Y.
January 1967

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CHAPTER 1

NITRITES, NITRATES, AND NITRO COMPOUNDS

THE ABSORPTION of numerous simple and complex phenols in dimethylformamide and in alkaline ethanol appears in Volume IIIA on pages 77-80. The determination of nitrofurazone and furazolidone has been extended since publication of Volume IIIA; they appear in that volume, on pages 270-2, as well as in this chapter. Nitrochloroform and nitrogen mustard appear in Volume IIIA as halogen compounds, on pages 492-3 and 520-1, respectively. A great deal of information about reading *m*-dinitrobenzene appears in Volume IIIA, on pages 10-3, as related to nitration of benzene for its determination.

Chloramphenicol, although a nitro compound, will appear in Vol. IVAA as an antibiotic. A method given in this chapter for glyceryl trinitrate by hydrolyzing and using the nitrous acid to diazotize sulfanilic acid and then coupling with *N*-(1-naphthyl)ethylenediamine is also applicable to *1-chloropropane-2,3-diol dinitrate*.

The red color that develops when sulfanilic acid is diazotized with isoamyl nitrite followed by coupling with *N*-(1-naphthyl) ethylenediamine is the basis for a determination of isoamyl nitrite.¹ The absorbance of samples that contain less than 0.002 mg. per ml. and are kept at 0° follows Beer's law. The color is stable after 30 minutes. The method for development of nitroglycerine with ferrous sulfate, page 10 of this volume, is also applicable to a 0.02-ml. sample of isoamyl nitrite.

Erythritol tetranitrate, *pentaerythritol tetranitrate* and *mannitol hexanitrate* are read in the infrared.² To determine *phenols* present in manufacture of nitrocyclohexane, pass the product through a column of aluminum oxide.³ Wash with ethyl ether to complete removal of nitrocyclohexane and cyclohexane. Elute with methanol and read at 355 $m\mu$ as the sum of nitrophenols, including picric acid. *Dinitrophenyl groups* react with potassium cyanide.⁴ The reaction is specific and is particularly applicable to dinitropesticides. Other pesticides do not interfere. A specific application to dinitro-*o*-cresol appears on page 51.

¹ Masao Maruyama and Yasuo Morotomi, *Takamine Kenkyusho Nempo* 11, 122-4 (1959).

² Carol Jonas, *J. Assoc. Offic. Agr. Chemists* 43, 259-61 (1960).

³ G. I. Romantsova, *Zavod. Lab.* 29, 1307-8 (1963).

⁴ Calvin Menzie, *J. Agr. Food Chem.* 6, 212-3 (1958).

Both *m*-dinitrobenzene (cf. Vol. IV, p. 16) and *sym*-trinitrobenzene (cf. Vol. IV, p. 20) are read in benzene as a red complex with sodium ethoxide.⁵ They are separable by chromatography on activated magnesium oxide.

Nitrofuran reacts with sodium hydroxide in aqueous solution to give a color appropriate for reading.⁶ Properly reduced in sodium salicylate solution, it shows $E_{1\%}^{1\text{cm}} = 430$ at 260 $m\mu$ and $E_{1\%}^{1\text{cm}} = 575$ at 360 $m\mu$.⁷ Nitrofuran derivatives such as *furadonine*, *furazolidone*, *furazidine*, and *furazoline* are also read in strongly alkaline solutions. If an easily hydrolyzed hydantoin or oxazolidinone ring is present, the alkali does not decompose the ring.⁸ For determination of *nitrofurazone*, *furazolidone*, *furaltadone*, *nitrofurantoin* and 1-[3-(5-nitro-2-furyl)allylidene-amino] *hydantoin* in alkaline solution, dimethylformamide or acetone are appropriate solvents.⁹

Nitrofurantoin is extracted with 95% ethanol and read at 386 $m\mu$.¹⁰ *Naphthazylene nitrate* is read in ethanol at 281 $m\mu$.¹¹

4-(2,4-Dinitroanilino)phenol is read directly in ethanol solution at 375.4 or 404.3 $m\mu$.¹² Beer's law applies over the range of 0.0058-0.025 gram per liter. The compounds used in preparing it, that is, 4-aminophenol and 1-chloro-2,4-dinitrobenzene, do not interfere. Determine 4,4''-Bis-(2-imidazolin-2-yl)-2-nitroterephthalanilide from the first extract of tissue with perchloric acid. Then extract into butanol. Re-extract with water. Activate at 285 $m\mu$ and read the fluorescence at 365 $m\mu$.¹³

ORGANIC NITRO COMPOUNDS

Organic nitro compounds can be quantitatively measured by an adaptation of the brown-ring test with ferrous sulfate.¹⁴ Nitrites and

⁵ K. Cruse and R. Mittag, *Z. anal. Chem.* **131**, 273-8 (1950).

⁶ V. Ariesan, C. Ionescu, M. Ariesan and F. Gagin, *Farmacia* (Bucharest) **8**, 397-400 (1960).

⁷ Henryk Marciszewski, *Dissertations Pharm.*, **11**, 321-6 (1959).

⁸ V. E. Egert, M. Simanska and S. Hillers, *Latvijas PSK Zinātn Akad. Vestis Kim. Ser.* **1961** (2), 190-240.

⁹ V. E. Egert, S. A. Giller, A. A. Lielgalve and M. V. Shimanskaya, *Izv. Akad. Nauk Latv SSR, Ser. Khim.* **5**, 531-40 (1963).

¹⁰ W. Zyzyński, *Acta Polon. Pharm.* **18**, 365-70 (1961).

¹¹ D. Halot, *Ann. Pharm. Franc.* **19**, 483-93 (1961).

¹² A. Demian and W. Schmidt, *Rev. Chim., Bucharest* **13**, 758 (1962).

¹³ M. A. Chirigos, J. M. Venditti and G. R. Fanning, *Cancer Chemotherap. Rev.* **32**, 1-5 (1963).

¹⁴ M. H. Swann and M. L. Adams, *Anal. Chem.* **28**, 1630 (1956).

thiosulfates interfere. Chromates, dichromates, sulfates, phosphates, chlorates, sulfites, acetates, and halogens do not. The procedure found under hexahydro-1,3,5-trinitro-s-triazine is also generally applicable to organic nitrates.

Many aromatic nitro compounds produce orange, red, or purple colors in dimethylformamide upon addition of tetraethylammonium hydroxide.¹⁵ The optimum range of concentration is 0.0002-0.005 mg. per ml. The amount of reagent added is critical as the color fades rapidly with a reagent of lower concentration. Water decreases the absorbance and ethyl acetate interferes. This method can be satisfactorily applied to the compounds listed in Table 1, less satisfactorily to most organic nitro compounds.

TABLE 1. SOME COMPOUNDS AMENABLE TO QUANTITATIVE DETERMINATION IN DIMETHYLFORMAMIDE BY TETRAETHYLAMMONIUM HYDROXIDE

Compound	Wave length, $m\mu$	Time of readings after addition of TEAH, min.	Av. absorbance ^a per γ per ml.	
4-Nitrobenzoyl-4'-nitrophenylhydrazine	660	1-2	0.066	0.0014
4,4'-Dinitrostilbene	596	20-40	0.053	0.0010
4,4'-Dinitrocarbanilide	556	1-2	0.189	0.0036
2-Nitrodiphenylamine	550	1-10	0.051	0.0001
2-Nitro-4-chloroaniline	510	1-3	0.057	0.0013
2-Nitro-4-chlorophenylurea	510	4-20	0.053	0.0012
<i>o</i> -Nitroaniline	504	1-3	0.068	0.0013
4-Nitro- <i>o</i> -phenylenediamine	503	1	0.197	0.0025
<i>o</i> -Nitrophenylurea	502	4-20	0.057	0.0010
<i>o</i> -Nitroxylidine	500	1-10	0.069	0.0019
4-Nitrophenylbiguanide	495	4-20	0.194	0.0026
4-Nitrocarbanilide	480	1-10	0.176	0.0031
4-Nitro-4'-chlorocarbanilide	480	1-5	0.145	0.0020
5-Nitro-2-aminoanisoie	478	1-20	0.193	0.0025

^a Light path, 1.3 cm.

Many nitro compounds are satisfactorily determined on 5-mg. samples by: (1) reduction with zinc and hydrochloric-acetic acid mixture; (2) Kjeldahl digestion; and (3) development with Nessler's reagent.¹⁶

¹⁵ Curt. C. Porter, *ibid.* 27, 805-7 (1955).

¹⁶ Marcello Piazzi, *Ann. chim. (Rome)* 51, 886-90 (1961).

Procedure—*By ferrous sulfate heptahydrate.* As reagent, dissolve 0.5 gram of ferrous sulfate heptahydrate in 25 ml. of water. With cooling, add 75 ml. of concentrated sulfuric acid.

Dilute a sample with water, acetone, or similar solvent so that an aliquot contains 0.5-2.5 mg. of nitrate. Make the sample slightly alkaline with a few drops of 0.4% sodium hydroxide solution and dry in an oven at 105°. Add 20 ml. of reagent, stopper, and let stand for 90 minutes with frequent agitation. Read at 525 $m\mu$ against a reagent blank.

By dimethylformamide and tetraethylammonium hydroxide. To a solution containing 0.005-0.1 mg. of nitro compound in 9.9 ml. of dimethylformamide, add 0.1 ml. of 10% tetraethylammonium hydroxide solution. Read at the proper wave length at the appropriate time after addition of reagent according to Table 1.

PRIMARY ALIPHATIC NITROPARAFFINS

Primary nitroparaffins are determined by the coupling reaction with *p*-diazobenzenesulfonic acid, carried out at pH 4.3¹⁷ (cf. Vol. IV, p. 1). Secondary nitroparaffins and 2-nitro-2-alkyl-1-alkanols do not interfere. 2-Nitro-2-alkyl-1,3-alkenediols react with the reagent. Thus the reaction is suitable for determination of nitromethane, nitroethane, nitropropane, etc.

The reaction product with nitromethane is 1-nitroformaldehyde *p*-sulfophenylhydrazone, with nitroethane, 1-nitroacetaldehyde *p*-sulfophenylhydrazone, and with nitropropane, 1-nitropropionaldehyde *p*-sulfophenylhydrazone.

Procedure—*By p-diazobenzenesulfonic acid.* To prepare the phosphate buffer, add 4 volumes of 1.04% monobasic sodium phosphate solution to 1 volume of 1.788% dibasic sodium phosphate solution. As a reagent, prepare a 0.1% potassium nitrite solution. Mix 7.2 grams of sulfanilic acid and 18 ml. of concentrated hydrochloric acid and dilute to 1 liter with water. Mix equal volumes of the potassium nitrite and the sulfanilic acid solutions.

To 1 ml. of sample solution containing up to 0.05 mg. of nitromethane or up to 0.08 mg. of nitroethane or up to 0.1 mg. of 1-nitropropane, and to a 1-ml. blank containing 10% methanol, add 1.4 ml. of the phosphate buffer and 0.6 ml. of 3% potassium hydroxide solution. With vigorous

¹⁷ Israel R. Cohen and A. P. Altshuller, *Anal. Chem.* **31**, 1638-40 (1959).

shaking, add 5 ml. of the reagent solution. Read, within 10 minutes, at 440 m μ for nitromethane and at 395 m μ for higher nitroparaffins.

PRIMARY AND SECONDARY ALIPHATIC NITRO COMPOUNDS

Primary aliphatic nitro compounds when hydrolyzed by sulfuric acid yield hydroxylamine.¹⁸ Oximes give the same reaction. For determination, the hydroxylamine is oxidized to nitrous acid with iodine. This is used to diazotize sulfanilic acid and is coupled with 1-naphthylamine to give a magenta color.¹⁹ The reaction has been satisfactorily applied to nitroethane, 1-nitropropane, 4-chloro- β ,2-dinitrostyrene, and ethyl- β -carboxy- δ -nitrovalerate at not over 0.005 mg. per ml.

Secondary nitro compounds yield nitrous acid directly on such hydrolysis.²⁰ The method as applied to diethylnitropinylate, 1-phenyl-2-nitropropane, and ethyl- β -hydroxy- γ -nitrocaproate, but omitting the oxidation with iodine, did not give the color. When oxidation was applied, they reacted like primary nitro compounds. No color was developed with typical tertiary nitro compounds, 2-nitro-2-methyl-1-propanol, and *tris*-(β -carboxymethyl)nitromethane, or with such aromatic nitro compounds as chloramphenicol, nitrobenzaldehyde, *p*-nitrophenylacetic acid, *p*-nitrophenylserinol, or dinitrophenol. An application is to filtrates from biosynthesis of chloramphenicol with *Streptomyces venezuelae*.

Procedure—To 1 ml. of sample, add 1 ml. of 0.5% sulfanilic acid solution in 35% acetic acid. Mix with 1 ml. of 1:2.5 sulfuric acid and heat in boiling water for 15 minutes. At this step, any nitrite in the original culture is removed and the nitro compound converted to hydroxylamine. Cool, and add 1.8 ml. of a buffer containing 35 grams of sodium acetate trihydrate in 100 ml. of water. Shake, and add 0.4 ml. of 1.3% iodine in glacial acetic acid. Shake, and allow to stand for 4 minutes to oxidize hydroxylamine to nitrous acid. Add 1 ml. of aqueous 2% sodium arsenite solution to remove excess iodine. Finally, add 1 ml. of 0.2% 1-naphthylamine hydrochloride in 35% acetic acid. Dilute to 10 ml. and mix. Read at 550 m μ . An appropriate standard for preparation of a calibration curve is nitroethane.

¹⁸ E. F. Degering, *An Outline of Organic Nitrogen Compounds*, University Lithoprinters, Ypsilanti, Mich. (1945).

¹⁹ Ralph B. Gwatkin, *Nature* **193**, 973-4 (1962).

²⁰ Ralph B. Gwatkin and D. Gottlieb, *J. Bact.* **71**, 328 (1956).

NITROMETHANE

Sodium 1,2-naphthoquinone-4-sulfonate reacts with nitromethane in alkaline solution to yield a violet complex. This is extracted with isoamyl alcohol and read at 585 $m\mu$. Beer's law is followed for 0.005-0.03 mg. of nitromethane and the reaction is specific.²¹ Excessive amounts of nitroethane, 1-nitropropane, 1-nitrobutane, and 1-nitro-2-methyl propane interfere. When nitromethane is present in concentrations of less than 5% in the presence of these compounds, the nitromethane must be separated out by an azeotropic distillation with methanol. By applying this method of separation, as little as 1 part of nitromethane in 10,000 parts of other nitroparaffins can be measured.

Sample—Air. Using a flow rate of 0.2 liter per minute, pass the air sample through an aeration train of three tubes, each containing 20 ml. of phosphate buffer as prepared for the procedure. Combine the buffer solutions and dilute so that a 1-ml. aliquot contains up to 0.03 mg. of nitromethane.

Separation of nitromethane from other nitroparaffins by azeotropic distillation. To a 1-2-gram sample, add 200 ml. of dry methanol. Reflux for 1 hour, using a fractionating column. Remove 50 ml. of the distillate at a reflux ratio of 30 to 1 and dilute to 100 ml. with water.

Procedure—To prepare the phosphate buffer, adjust a 20% dibasic potassium phosphate solution to pH 9.5 with a saturated solution of tripotassium phosphate. Dilute a 1-ml. neutral sample containing up to 0.03 mg. of nitromethane to 100 ml. with water. To a 1-ml. aliquot, add 10 ml. of phosphate buffer and 1 ml. of freshly prepared 0.1% sodium 1,2-naphthoquinone-4-sulfonate solution and mix. After 15 minutes at room temperature, add 5 ml. of isoamyl alcohol that has been saturated with phosphate buffer. Mix and centrifuge. Read a portion of the upper isoamyl alcohol phase at 585 $m\mu$ against a water blank similarly treated.

NITROETHANE

For determination by *p*-diazobenzenesulfonic acid, see primary aliphatic nitroparaffins page 4.

²¹ Lawrence R. Jones and John A. Riddick, *Anal. Chem.* 28, 1493-5 (1956).

1-NITROPROPANE AND RELATED MATERIALS

Primary nitroparaffins react with nitrous acid to give a red-orange color. If the product of the reaction is extracted with sodium hydroxide, 1-nitropropane will form sodium propylnitrolate that has a strong absorbance at 330 $m\mu$.²²

Beer's law is followed for 1-nitropropane for 0.002-0.05 mg. per ml. The method is applicable to 1-nitropropane, 2-nitropropane, 2,2-dinitropropane, 2-nitro-1-butanol, 2-nitro-2-methyl-1,3-propanol, 2-nitro-2-methyl-1,3-propanediol, 2-nitro-2-ethyl-1,3-propanediol and *tris*(hydroxymethyl)nitromethane.

Procedure—To a 2-ml. aliquot of aqueous solution of the sample, add a potassium hydroxide pellet of approximately 0.1 gram. Add about 0.25 gram of potassium nitrite and 0.6 ml. of 1:1 sulfuric acid. Shake with 10 ml. of diethyl ether. To the ether layer, add 3 ml. of water and 4-5 drops of a saturated sodium hydroxide solution. Shake, and read the aqueous layer at 330 $m\mu$ after 5 minutes.

NITRO ALCOHOLS

In the presence of excess alkali, nitro alcohols decompose to form formaldehyde and a nitroparaffin, while amino alcohols will not so decompose. The nitro alcohol is determined indirectly by measuring the formaldehyde formed, using chromotropic acid reagent.²³ By this method, 0.001-0.1 mg. of nitro alcohol may be determined, and 1 part of nitro alcohol in 1000 parts of amino alcohol may be measured.

The method is specific for nitro alcohols in the absence of formaldehyde or other compounds that hydrolyze to formaldehyde in alkaline solution. Factors that affect the determination of formaldehyde with chromotropic acid are also applicable in this reaction (Vol. IIIA, p. 252). The interference of amino hydroxy compounds is eliminated by refluxing with alkaline bisulfite solution. 2-Nitro-1-butanol and 2-nitro-2-methyl-1-propanol decompose to yield one molecule of formaldehyde each. 2-Nitro-2-methyl-1,3-propanediol, 2-nitro-2-ethyl-1,3-propanediol and *tris*(hydroxymethyl)nitromethane decompose to yield two molecules of formaldehyde per molecule of nitro alcohol.

²² A. P. Altshuller and I. R. Cohen, *Anal. Chem.* **32**, 881-2 (1960).

²³ Lawrence R. Jones and John A. Riddick, *Anal. Chem.* **28**, 254-5 (1956).

Procedure—Dilute a sample containing 0.001-0.1 mg. of nitro alcohol, such as 2-nitro-2-methyl-1-propanol, to 100 ml. with water. To a 1-ml. aliquot in a Lewis-Benedict tube, add 1 ml. of 2% sodium hydroxide solution. Mix and store at 25° for 5 minutes. Add 1 ml. of 2% chromotropic acid solution, dilute to 12.5 ml. with concentrated sulfuric acid, and mix well. Heat at 100° for 10 minutes and adjust the volume to 12.5 ml. with sulfuric acid. Read at 580 m μ against a reagent blank.

In the presence of α -aminohydroxy compounds. Dilute a 10-gram sample to 100 ml. with water and mix. To a 10-ml. aliquot, add 10 grams of sodium bisulfite crystals, 10 ml. of 20% sodium hydroxide solution, and 55-60 ml. of water. Reflux for 30 minutes, cool to 25°, and dilute to 100 ml. with water. Develop a 2-ml. aliquot as above.

3-NITROPROPANOIC ACID

3-Nitropropanoic acid is effectively determined in biological samples by displacement of the nitro group at pH 9.5, followed by reaction of the nitrite ion with a sulfanilic acid-1-naphthylamine hydrochloride reagent.²⁴ An excess of formaldehyde aids in the nitro group replacement and prevents free amino acids from reacting with the nitrite ion in acid solution.

A borax-sodium hydroxide buffer is used in preference to a saturated borax buffer. Ethanol, which is added to prevent precipitation caused by formaldehyde reactions, also increases the color stability and intensity as well as shifting the absorption maximum from 525 to 520 m μ . The control of pH is critical in displacement of the nitrite by base. Leaf samples are read directly after dilution to prevent interference of plant pigments. Biological samples containing large quantities of urea, free amino acids, or carbohydrates must be extracted with ether to isolate the nitropropanoic acid.

Sample—*Creeping indigo leaf.* Digest a 2-gram sample of leaf meal in 50 ml. of 1:110 hydrochloric acid for 1 hour by boiling gently. Cool, and adjust to pH 6 with 4% sodium hydroxide solution. Dilute to 100 ml., shake, and allow the solids to settle overnight. Centrifuge, or filter if cloudy.

²⁴ Hiromu Matsumoto, A. M. Unrau, J. W. Hylin and Barbara Temple. *Anal. Chem.* 33, 1442-4 (1961).

Biological samples containing large concentrations of urea, free amino acids, or carbohydrates. Follow the procedure for creeping indigo leaf samples. Strongly acidify the supernatant liquid and continuously extract in a liquid-liquid extractor with ether for at least 5 hours. Remove the ether and dissolve the residue in 5 ml. of water.

Procedure—To prepare the nitrate reagent, dissolve 0.6 gram of sulfanilic acid in 100 ml. of 12% acetic acid solution. Dissolve 0.6 gram of 1-naphthylamine hydrochloride in 100 ml. of 12% acetic acid solution and mix the two solutions. To prepare the borax-sodium hydroxide buffer, dissolve 10 grams of borax in 600 ml. of water and add 200 ml. of 0.8% sodium hydroxide solution. Adjust the pH to 9.5 with either 4% sodium hydroxide solution or 1:110 hydrochloric acid, and dilute to 2 liters.

To a 3-ml. aliquot of sample, add 1 ml. of 10% formaldehyde solution prepared by diluting 20 ml. of 37% formaldehyde solution to 75 ml. with water. Add 5 ml. of the borax-sodium hydroxide buffer and heat at 120° for at least 45 minutes. Add 30 ml. of water and cool in ice. Add 2 ml. of nitrite reagent followed immediately by 2 ml. of 1:1 hydrochloric acid. This solution should be at pH 1 to 1.5. After 10 minutes, add 2 ml. of 8% sodium acetate solution. If the solution is not at pH 2 to 2.5, adjust accordingly. Add 35 ml. of 95% ethanol and dilute to 100 ml. with ethanol. Read at 525 $m\mu$ against a reagent blank.

NITROGLYCOL

Methods for glyceryltrinitrate are applicable.²⁵

NITROGLYCERIN, GLYCERYL TRINITRATE

When glyceryl trinitrate is saponified in alkaline solution, the resulting nitrous acid can be determined. An appropriate method is to use it to diazotize sulfanilic acid or procaine and couple with *N*-(1-naphthyl)-ethylenediamine.²⁶ For other methods, see Volume IIA, pages 699-701.

Glyceryl trinitrate can be determined in the presence of pentaerythritol tetranitrate by the differences in rate of hydrolysis by sodium

²⁵ Tatsuo Akisada, *Japan Analyst* **12**, 443-6 (1963).

²⁶ F. K. Bell, J. J. O'Neill and R. M. Burgison, *J. Pharm. Sci.* **52**, 637-9 (1963); F. K. Bell, *J. Pharm. Sci.* **53**, 752-5 (1964).

hydroxide of the two compounds²⁷ (cf. Vol. IV, p. 8). Glyceryl trinitrate is completely hydrolyzed in 1 hour at 20°, while pentaerythritol tetranitrate remains stable. Color is developed from the diazotized hydrolysate with 1-naphthylamine. The calibration curve is prepared with sodium nitrite.

Ferrous sulfate is another reagent for development of color with nitroglycerin.²⁸

After separation of the glyceryl trinitrate by column chromatography, the familiar reaction of nitrate with phenoldisulfonic acid is used to develop the color.²⁹ At 7.90 μ , nitroglycerin in carbon disulfide follows Beer's law in the range of 0.36-1.8 mg. per ml.³⁰

Procedure—*By 1-naphthylamine. Tablets.* Shake a powdered sample containing approximately 1 mg. of glyceryl trinitrate at intervals with 20 ml. of 0.4% sodium hydroxide solution at 20° for 2 hours. Dilute to 50 ml. and centrifuge. To a 2-ml. aliquot, add 8 ml. of water, 1 ml. of 0.5% sulfanilic acid solution, 10 ml. of 1:5 hydrochloric acid, and 1 ml. of 0.5% 1-naphthylamine solution in a 1:4 mixture of acetone-1:110 hydrochloric acid. Dilute to 25 ml. with water and read at 530 $m\mu$ after 30 minutes.

*Air.*³¹ Bubble 3 liters of air through 5 ml. of 4% sodium hydroxide solution in 20% ethanol. Heat at 100° for 30 minutes. Add 2 ml. of 1:3 hydrochloric acid and 0.5 ml. of 0.6% sulfanilic acid in 1:5 hydrochloric acid. Mix, and set aside for 5 minutes. Add 0.5 ml. of a 0.6% solution of 1-naphthylamine in 1:10 hydrochloric acid and 0.5 ml. of 28% aqueous solution of sodium acetate trihydrate. Dilute to 10 ml., let stand for 10 minutes, and read at 530 $m\mu$. Multiply the amount of nitrite present by 9.77 to give either nitroglycerin or nitroglycol.

By ferrous sulfate. Extract a 50-mg. sample with ten successive 5-ml. portions of chloroform. Evaporate the solvent from the extracts at 35° *in vacuo*. Take up the residue in ethanol to contain about 1% of nitroglycerin.

To 1.5 ml. of sample, add 1.5 ml. of glacial acetic acid. Dilute 100 ml. with 0.5% solution of ferrous sulfate heptahydrate in 75% sulfuric acid.

²⁷ M. Halse, *Medd. Norsk Farm. Selskap* **16**, 166-9 (1954).

²⁸ A. F. Fursov, *Aptekhn. Delo* **10**, No. 1, 9-13 (1961).

²⁹ John R. Hohmann and Joseph Levine, *J. Assoc. Official Agr. Chemists* **47**, 471-3 (1964).

³⁰ George Schwartzman, *ibid.* **39**, 254-5 (1956).

³¹ Tatsuo Akisada, *Japan Analyst* **12**, 443-6 (1963).

Add 3 mg. of sodium sulfite and shake for 3 minutes. Read at 510 $m\mu$. The color is stable for at least 2 hours.

By N-1-naphthylethylenediamine. Tablets. Shake a ground tablet vigorously with 25 ml. of water for 5 minutes. Filter, and take an aliquot equivalent to about 0.005 mg. of glyceryl trinitrate. Dilute with water to 1 ml. and add 4 ml. of 1% solution of strontium hydroxide octahydrate. Heat at 50° for 15 minutes and cool. Add 1 ml. of 0.3% procaine hydrochloride solution, 1 ml. of 1:2 hydrochloric acid, and 1 ml. of 0.1% N-1-naphthylethylenediamine solution. Dilute with water to 10 ml. and let stand for 5 minutes. Read at 550 $m\mu$ and subtract a reagent blank.

By phenoldisulfonic acid. Tablets. Prepare a phenoldisulfonic acid reagent by heating 15 grams of colorless phenol with 100 ml. of concentrated sulfuric acid at 100° for 5 hours. Weigh not less than 20 tablets, and finely powder. Dissolve a portion equivalent to about 30 mg. of glyceryl trinitrate in water, and dilute to 100 ml.

Mix 3 grams of acid-washed Celite 545 with 2 ml. of water and pack in a 6-cm. \times 5-mm. chromatographic column over glass wool. The tube has a 25 \times 2.5-cm. reservoir. Mix 3 grams of Celite and 2 ml. of the sample solution and pack in the column. Pass 70 ml. of isooctane through the column and collect the eluate in a separatory funnel containing 2 ml. of the phenoldisulfonic acid reagent and 1 ml. of glacial acetic acid. Shake vigorously for 3 minutes and let stand for 25 minutes. Extract successively with 20, 20, 10, and 10 ml. of water. Add 10 ml. of concentrated ammonium hydroxide to the combined extracts and dilute to 100 ml. Filter if necessary, discarding the first 5 ml. of filtrate.

At this point, prepare an inorganic standard. This consists of 0.1 gram of sodium nitrate or potassium nitrate dissolved in about 1 ml. of water and diluted to 100 ml. with glacial acetic acid. Mix 4 ml. of phenoldisulfonic acid with 2 ml. of the standard nitrate solution and let stand for 15 minutes. Dilute with 100 ml. of water and add 20 ml. of concentrated ammonium hydroxide. Cool, and dilute to 200 ml. Read the sample and standard at 408 and 600 $m\mu$ against a blank of acetic acid similarly treated. Calculate as follows:

$$[A_{408} - A_{600}/A'_{408} - A_{600}] \times C \times R \times$$

(average weight per tablet/weight of sample) = mg. per tablet

where C is the concentration of inorganic standard, and R is the factor for conversion of the standard to glyceryl trinitrate, 0.749 for KNO_3 , 0.891 for NaNO_3 .

In carbon disulfide. Tablets. Powder the samples and weigh a portion equivalent to approximately 50 mg. of nitroglycerin. Add 15 ml. of water and mix thoroughly. Extract with one 20-ml. portion and two 10-ml. portions of carbon disulfide, filtering each extract through dry filter paper. Dilute the combined filtrates to 50 ml. with carbon disulfide and mix thoroughly. Weigh a sample of 9% nitroglycerin absorbed on lactose and standardized by the U.S.P. procedure. Treat the standard as the sample. Compare the recorded spectra of the sample and standard from 2-15 μ to determine the identity of the sample and determine the baseline absorbance of each at 7.90 μ .

ORGANIC NITRATES AND NITRAMINES

In the presence of sulfuric acid, organic nitrates and aliphatic nitramines liberate their nitrogen as nitric oxide, which, in turn, reacts with ferrous ion to produce a stable red-violet colored complex.³² The reaction is not specific for -ONO₂ groups, as -N-NO₂ groups will also react. Beer's law is followed up to 2.6 milliequivalents of nitric oxide per mole of sample per 100 ml. of solution.

This procedure has been tested for potassium nitrate, *nitroglycerin*, *pentaerythritol tetranitrate*, *triethyleneglycol dinitrate*, *N-methyl-N-nitro-2,4,6-trinitroaniline*, *hexahydro-1,3,5-trinitro-s-triazine*, *octahydro-1,3,5,7-tetranitro-s-tetrazine* and *nitroguanidine*. All compounds give the same straight-line curve with the exception of hexahydro-1,3,5-trinitro-s-triazine and octahydro-1,3,5,7-tetranitro-s-tetrazine. Both compounds readily hydrolyze in the presence of sulfuric acid, producing formaldehyde, which interferes with the formation of the ferrous ion complex and accounts for the lower slopes of the Beer's law curves.

Since the slopes of the curves of these two compounds differ significantly, mixtures of the compounds may be analyzed without prior separation, using a ferrous ammonium sulfate reagent.³³ Because of solubility, the maximum concentration of a sample solution at room temperature is 40 mg. per 100 ml. Nitrites and thiosulfates interfere. Chromates, dichromates, sulfates, phosphates, chlorates, sulfites, acetates, and halogens do not interfere.

³² Mario A. Laccetti, Stanley Semel and Milton Roth, *Anal. Chem.* **31**, 1049-50 (1959).

³³ Stanley Semel, Mario A. Laccetti and Milton Roth, *Anal. Chem.* **31**, 1050-2 (1959).

Procedure—General. To prepare the ferrous sulfate solution, add 250 ml. of water to 10.5 grams of anhydrous ferrous sulfate. Bring to a rapid boil with stirring. Cool to room temperature. With the solution in a cold water bath, slowly add 600 ± 50 ml. of concentrated sulfuric acid, cool to room temperature, and dilute to 1 liter with the same acid.

Dilute a 0.25-gram sample to 250 ml. with acetone. Evaporate the acetone from an aliquot containing approximately 2 milliequivalents of nitric oxide that would be formed from the sample, using a stream of dry air. Add 50 ml. of concentrated sulfuric acid. Shake to dissolve. Add 50 ml. of ferrous sulfate reagent at 10-15°, stopper, and cool under running water. Swirl occasionally, keeping the temperature between 25 and 30°. Read at 525 $m\mu$, after 45 minutes, against a blank prepared by mixing equal volumes of ferrous sulfate reagent and concentrated sulfuric acid.

Organic nitrates and aliphatic nitramines may be identified by the following equation:

$$M = \frac{NS}{0.413A}$$

in which M is the molecular weight of the sample; S is the sample weight, mg/100 ml. of solution; N is moles of nitric oxide released per mole of sample; and A is the absorbance of the sample at 525 $m\mu$ with a 1-cm. cell.

Simultaneous determination of hexahydro-1,3,5-trinitro-s-triazine and octahydro-1,3,5,7-tetranitro-s-tetrazine by ferrous ammonium sulfate. To prepare the ferrous ammonium sulfate reagent, dissolve 100 grams of hydrated ferrous ammonium sulfate in 500 ml. of water. Slowly add 50 ml. of concentrated sulfuric acid and cool to room temperature. Add 1 gram of iron filings and allow the hydrogen to escape. Dilute to 1 liter with water and cool to 15-25°.

Dissolve a 0.4-gram sample in 150 ml. of acetone and dilute to 250 ml. with acetone. Evaporate the acetone from a 25-ml. aliquot containing up to 35 mg. of hexahydro-1,3,5-trinitro-s-triazine per 100 ml. and up to 50 mg. of octahydro-1,3,5,7-tetranitro-s-tetrazine per 100 ml., using a stream of dry air. To the residue, add 90 ml. of concentrated sulfuric acid. Stir, and cool to 20°. Slowly add 10 ml. of ferrous ammonium sulfate reagent. Swirl, and let stand at 20° for 45 minutes, swirling occasionally. Read at 525 $m\mu$ against a blank prepared by mixing equal volumes of reagent and concentrated sulfuric acid. The absorbances of the two compounds are additive.

TETRANITROMETHANE

The reaction of hydrazine with tetranitromethane produces the nitro-form ion, which absorbs at 350 $m\mu$.³⁴

Procedure—*Gas*. As an absorbing solution, dissolve 2.5 grams of sodium hydroxide and 0.3 gram of hydrazine sulfate in 250 ml. of water and dilute to 1 liter with 95% ethanol. Draw a measured volume of gas through about 10 ml. of this solution in an efficient absorber at a rate of 0.5-1 liter per minute. Dilute to 25 ml. with water and read at 350 $m\mu$.

Solutions. As reagent, dissolve 10 grams of hydrazine sulfate in 50 ml. of water. Mix with 20 grams of potassium hydroxide in 30 ml. of water. Filter, and dilute to 100 ml. with water. Add this reagent to a measured volume of sample in such an amount that the hydrazine to tetranitromethane ratio is 2:1 to 5:1. Dilute to a known volume and read at 350 $m\mu$.

MANNITOL HEXANITRATE

Mannitol hexanitrate is determined by the reaction of the nitrate group with phenoldisulfonic acid.³⁵ Either potassium nitrate or sodium nitrate is used as the standard. Beer's law is followed. Phenobarbital does not interfere.

Procedure—To prepare the reagent, add 30 ml. of concentrated sulfuric acid and 15 ml. of fuming sulfuric acid to 5 grams of phenol. Heat at 100° for 2 hours.

Dilute a sample containing up to 30 mg. of mannitol hexanitrate to 50 ml. with glacial acetic acid. Filter, discarding the first 5 ml. To a 1-ml. portion of the filtrate, add 2 ml. of phenoldisulfonic acid reagent. After 15 minutes, add 60 ml. of water and approximately 10 ml. of concentrated ammonium hydroxide. Cool to room temperature in an ice bath. Dilute to 100 ml. with water and read at 408 $m\mu$ against a reagent blank.

The concentration is given by:

$$\% \text{ mannitol hexanitrate} = \frac{(A_{\text{sample}} \times \text{mg. standard})}{(A_{\text{standard}} \times \text{wt. charge})} \times 100$$

If sodium nitrate is used as the standard, the percentage of mannitol

³⁴ Donald L. Glover and Shirley D. Landsman, *Anal. Chem.* **36**, 1690-1 (1964).

³⁵ Evelyn Sarnoff, *J. Assoc. Offic. Agr. Chemists* **38**, 637-8 (1955).

hexanitrate is multiplied by 0.8865; if potassium nitrate is used, the factor is 0.7456.

NITROCELLULOSE

Nitrocellulose forms a yellow color by reaction with alkali in the presence of acetone.³⁶ By this method, nitrocellulose can usually be determined directly in lacquer vehicles, since solvents, most plasticizers, and coating resins do not interfere. Certain rosin products, phenol-aldehyde condensates, and free aldehydes interfere slightly. Free aldehyde interference can be eliminated by oven-drying the sample aliquot at 105°. Beer's law is followed up to 40 mg.

A modification of the well-known brown-ring test for nitrates with ferrous sulfate and sulfuric acid allows the quantitative determination of nitrocellulose in samples of high acid concentration.³⁷ Interference is encountered from nitrites and thiosulfates, while chromates, dichromates, sulfates, phosphates, chlorates, sulfites, acetates, and halogens can be tolerated. Nitrocellulose cannot be determined in lacquer coatings if the oil or plasticizer present is of the type that would interfere.

Procedure—Lacquers. Dissolve a 2-ml. clear sample in acetone and dilute to 25 ml. with acetone. Dilute an aliquot containing up to 40 mg. of nitrocellulose to 10 ml. with acetone. Add 10 ml. of 10% potassium hydroxide solution and a boiling stone. Reflux for 1 hour at 100°. Cool rapidly to room temperature and dilute to 50 ml. with a 2:1 acetone-water mixture. Filter if cloudy and read at 425 m μ . If the sample is pigmented and a clear sample cannot be obtained by centrifuging, prepare a blank from a duplicate aliquot, eliminating the alkali, and dilute to 50 ml. with acetone only.

By ferrous sulfate. As a reagent, dissolve 0.5 gram of ferrous sulfate heptahydrate in 25 ml. of water and slowly add, with cooling, 75 ml. of concentrated sulfuric acid. Cool to room temperature. Dilute a sample with acetone so that an aliquot contains 0.5-2.5 mg. of nitrate. Make the solution slightly alkaline with a few drops of 0.4% sodium hydroxide solution, and dry in an oven at 60°, or dry with a current of air. Add 20 ml. of ferrous sulfate reagent, stopper, and let stand for 90 minutes with frequent agitation. Read at 525 m μ against a reagent blank.

³⁶ M. H. Swann, *Anal. Chem.* **29**, 1504-5 (1957).

³⁷ M. H. Swann and M. L. Adams, *Anal. Chem.* **28**, 1630 (1956).

AROMATIC NITRO COMPOUNDS

Formamidine sulfinic acid, thiourea dioxide, reduces aromatic nitro compounds under alkaline conditions to primary aromatic amines. The resulting aromatic amines are determined in the range of 5-50 mg. per liter by reading the red azo dye formed by diazotization and coupling with 1-amino-8-hydroxynaphthalene 2,4-disulfonic acid, Chicago acid.³⁸ Excess formamidine sulfinic acid is destroyed by heating in acid solution at 50°.

The procedure is applicable to a large number of compounds, as is shown in Table 2. The nitro compound is determined in the range of

TABLE 2. WAVE LENGTHS FOR READING NITRO COMPOUNDS
DEVELOPED AS AMINES WITH CHICAGO ACID

<i>Nitro compound</i>	<i>Amino compound</i>	<i>Wave length Max. abs. mμ</i>
Nitrobenzene	Aniline	520
<i>o</i> -Nitrotoluene	<i>o</i> -Toluidine	535
<i>p</i> -Nitrotoluene	<i>p</i> -Toluidine	550
<i>o</i> -Nitrochlorobenzene	<i>o</i> -Chloroaniline	520
<i>p</i> -Nitrochlorobenzene	<i>p</i> -Chloroaniline	530
3,4-Dichloronitrobenzene	3,4-Dichloroaniline	530
2,5-Dichloronitrobenzene	2,5-Dichloroaniline	520
2-Chloro-4-nitrotoluene	2-Chloro-4-aminotoluene	520
4-Chloro-2-nitrotoluene	4-Chloro-2-aminotoluene	520
1-Nitronaphthalene	1-Naphthylamine	550
<i>o</i> -Nitrobiphenyl	<i>o</i> -Aminobiphenyl	540
<i>p</i> -Nitrobiphenyl	<i>p</i> -Aminobiphenyl	540
<i>p</i> -Nitroanisole	<i>p</i> -Anisidine	540
<i>p</i> -Nitrophenetole	<i>p</i> -Phenetidine	550
4-Methyl-2-nitroanisole	Cresidine	560
<i>m</i> -Nitroacetanilide	<i>m</i> -Aminoacetanilide	520
<i>p</i> -Nitroacetanilide	<i>p</i> -Aminoacetanilide	520
<i>m</i> -Dinitrobenzene	<i>m</i> -Phenylenediamine	520
2,4-Dinitrotoluene	2,4-Tolylenediamine	520

0.01-0.15 mg. The water used should contain less than 10 ppm. of iron. Before use, glassware should be soaked in 5% trisodium phosphate solution for several hours and rinsed in water. Corex cuvetts are washed in 2:3 nitric acid to avoid retention of nitrous acid on the walls of the equipment.

³⁸ W. H. Koniecki and A. L. Lynch, *Anal. Chem.* **30**, 1134-7 (1958).

Sample—Urine. To purify formamidine sulfinic acid, dissolve in 5 parts of 1.1% sodium bisulfite solution at 60-63°. Clarify, and recrystallize slowly with agitation at 10°. Dry the filter cake immediately at 60°. To prepare the reagent for color development, dissolve 0.5 gram of 1-amino-8-naphthol-2,4-disulfonic acid in 1 ml. of 2:3 hydrochloric acid and 45 ml. of water. Dilute to 50 ml. with water. Store in the dark in a refrigerator. Do not use after 3 days. As a buffer, dissolve 320 grams of sodium acetate trihydrate in 1 liter of water.

For each 5 ml. of sample, add 3 ml. of 10% sodium carbonate solution and 20-25 mg. of formamidine sulfinic acid. Dissolve by swirling. Stopper loosely and heat for 15-20 minutes at 50° with occasional swirling. Add 1.5 ml. of 1:9 hydrochloric acid while swirling, stopper loosely, and heat for 5-10 minutes at 50° to destroy excess reducing reagent. Cool to 0-5° in ice water for at least 10 minutes. Add 1.5 ml. of 1:9 hydrochloric acid. Mix rapidly to complete the destruction of excess formamidine sulfinic acid. Add 10 ml. of water and mix.

Diazotize at 0-5° with 1 ml. of 3% sodium nitrite solution. After 3-5 minutes, destroy excess nitrous acid by adding 1 ml. of 10% sulfamic acid solution. Mix well, let stand at 0-5° for 18-20 minutes, and test for nitrous acid by spotting on cadmium iodide-starch paper. If the test is positive, add 1 ml. more of the 10% sulfamic acid solution and let stand for 20 minutes. Divide the solution into two equal parts. To one portion, add 0.5 ml. of Chicago acid reagent. To both portions, add 5 ml. of sodium acetate buffer, and swirl. Heat each at 50° for 15-20 minutes and dilute each portion to 25 ml. with water. Read the portion containing the acid against the other portion within 4 hours at the wave length indicated in Table 2.

NITROBENZENE

Nitrobenzene in aqueous solution is reduced to aniline with sodium hydrosulfite. This is then diazotized and coupled with 2-naphthol-3,6-disulfonic acid, R-salt.³⁹ Nitrobenzene in acid aqueous solution can be reduced to aniline by zinc. The solution is neutralized and oxidized with sodium hypochlorite to form *p*-aminophenol. This reacts with phenol to give indophenol for reading at 607 m μ . The method will detect 0.0013 mg. in a 10-ml. sample.⁴⁰ Also see Volume IV, page 11.

³⁹ M. T. Golubeva, *Lab. Delo* 10, 6-8 (1964).

⁴⁰ Ch'u-Liang Chen, *Hua Hsüeh Shih Chieh* 539-40 (1959).

Procedure—Adjust the volume of the aqueous sample to 250 ml. according to the concentration in the original. Add 5 ml. of 10% sodium hydroxide solution and distil 50 ml. To 10 ml. of the distillate, and to known standards, add 0.2 ml. of fresh 1% sodium hydrosulfite solution. Heat at 100° for 10 minutes. Cool, and add 2 ml. of 1:10 hydrochloric acid and 1 ml. of 3.5% sodium nitrite solution. After 10 minutes, add 3 ml. of 10% sodium carbonate solution and 0.4 ml. of 0.17% solution of R-salt. A salmon-pink develops in 30-60 minutes.

1-CHLORO-2-NITROBENZENE

After extraction from the sample with benzene, 1-chloro-2-nitrobenzene is reduced, diazotized and coupled with *N*-(1-naphthyl)ethylenediamine.⁴¹ Beer's law is followed and 0.01 ppm. can be measured.

Procedure—*Pineapple*. As a sample, cut opposite longitudinal wedges from at least four pineapples into small pieces. Mix, and weigh 150 grams. Blend with 100 ml. of water and 150 ml. of benzene until the sample is homogenized. Centrifuge at 1500 rpm. for 10 minutes and siphon off 100 ml. of the supernatant liquid. Place a glass wool plug at the bottom of a 125-ml. open-top cylindrical separatory funnel or a similar chromatographic column and add 5 grams of a 2:1 mixture of Attapulugus clay and Celite in 20 ml. of benzene. Apply suction until the benzene reaches the top of the column.

Pass the sample through the column to remove pigments. Collect the extract and wash the column with 60 ml. of benzene. Add an acid-washed boiling stone, cover, and concentrate to 10 ml. at a low heat. Add 10 ml. of 95% ethanol, 10 ml. of 1:10 hydrochloric acid, and 0.2 gram of zinc powder. Cover, digest, and concentrate to 5 ml. with low heat. Filter, and wash to a volume of 20 ml. Add 1 ml. of 1% sodium nitrite solution and let stand for 10 minutes. Add 1 ml. of 10% ammonium sulfonate solution and stopper. Invert to remove all nitrite from the neck of the flask. Let stand for 10 minutes with occasional shaking to remove evolved nitrogen. Add 1 ml. of 1% *N*-(1-naphthyl)ethylenediamine dihydrochloride solution and dilute to 200 ml. Mix, let stand for 20 minutes, and read at 540 m μ .

⁴¹ H. Y. Young, *J. Agr. Food. Chem.* **8**, 213-4 (1960).

TERRACLOR, PCNB, PENTACHLORONITROBENZENE

The alkaline hydrolysis of pentachloronitrobenzene produces potassium nitrite, which is used to diazotize procaine hydrochloride. The diazotized product is coupled with 1-naphthylamine to give a magenta-colored solution, which is read at 525 m μ .⁴² This reaction is applicable to concentrations in the range of 0.01-0.05 mg.; as little as 0.02 ppm. may be determined. The maximum color development is in the range of pH 2 to 2.5. Pentachloronitrobenzene is separated from samples by chromatography on varied media, depending upon the fat, oil, and wax content of the sample. Tetrachloronitrobenzene interferes.

Samples—Specially purified petroleum ether is usually required. The purity must be such that 800 ml. concentrated to 5 ml. will not give a reagent blank equivalent to more than 3.6 ± 0.8 micrograms of PCNB. Also, the addition of 50 micrograms of PCNB in purified petroleum ether to such a 5-ml. concentrate shall not deviate by more than $\pm 10\%$ from the absorbance of such a standard to which the concentrate has not been added.

To purify, wash successively three times with concentrated sulfuric acid, once with water, once with 2% sodium bicarbonate solution, and thereafter with water until the washings are neutral. Dry with anhydrous sodium sulfate and filter. Distil the filtrate through a 400-mm column of glass beads as a 4-liter batch, discarding the first and last 100-ml. cuts. Store in glass.

Soil. Shake 100 grams of air-dried sample with 200 ml. of petroleum ether for 1 hour. Let the solids settle and decant the supernatant layer through prewashed glass wool. Collect 100 ml. of filtrate. Perform preliminary analyses of similar aliquots of the extracts of treated soils, control soil blanks, and control soils containing known amounts of PCNB. If the recovery standard is low, it may be due to sulfur in the soil. If the values are high, a cleanup with pretreated activated Florasil is indicated.

⁴² Henry J. Ackermann, Helen A. Baltrush, Herman H. Berges, Darwin O. Brookover and Bernard B. Brown, *J. Agr. Food Chem.* **6**, 747-50 (1958); C. A. Bache and D. J. Lisk, *ibid.* **8**, 459-60 (1960); A. K. Klein and R. J. Gajan, *J. Assoc. Agr. Chemists* **44**, 712-9 (1961); Henry J. Ackermann, Louis J. Carbone and Edward J. Kuchlar, *ibid.* **11**, 297-300 (1963); P. Bracha, *J. Agr. Food Chem.* **11**, 297-300 (1964).

Sulfur cleanup. Coil 16 Band S copper wire around a 5-mm. glass rod to give 12 loops. Clean immediately before use in successively concentrated nitric acid, water, acetone, and petroleum ether. Dilute or concentrate an appropriate sample to 25 ml. Reflux with the copper wire, replacing the wire if it becomes discolored by cupric sulfide. Concentrate the extract to 5 ml. and proceed.

Florasil cleanup. To prepare the Florasil, add 120 grams of 60-100 mesh, 110° or 260° grade, to 700 ml. of 1:1 hydrochloric acid. Stir for exactly 1 minute and immediately filter with vacuum on fritted glass. Wash with water without agitating the filter cake, until the washings are neutral. Dry overnight at 120°. Activate at 650° for 2 hours. Transfer to glass-stoppered bottles and store at 120° until used.

Add a 100-mm. column of the Florasil to a 50 × 400-mm column. Top it with 20 mm. of anhydrous granular sodium sulfate. Prewash with 75 ml. of petroleum ether, leaving 2 mm. above the column. Add the sample, concentrated to 25 ml., if necessary, to the column. Elute it with 6% of diethyl ether in petroleum ether and concentrate the eluate to 5 ml.

Lettuce, green beans, peppers, potatoes, celery, tomatoes. Clean the sample and cut into small pieces. Blend 200 grams with 250 to 300 ml. of methylene dichloride with a slow blending speed to minimize emulsion formation. Separate the extracts from the pulp in a basket centrifuge lined with filter paper. Reserve any aqueous material in the basket and re-extract the pulp and paper matrix. Combine all aqueous layers and wash with methylene dichloride, adding the washes to the extracts. Dry the extract with sodium sulfate. Evaporate the solvent at 100° and remove the last traces of solvent with a stream of air. To the residue, add 25 ml. of petroleum ether and reflux for 15 minutes.

Prepare a chromatographic column 30 × 280 mm., with a medium-porosity fritted-glass disk having a vacuum sidearm below the disk and $\text{F } 24/40$ joint at the base. As an adsorbant, heat magnesium oxide at 200° for 1 hour. Cool in a sealed jar. Grind 100 grams with 6 ml. of water, seal, and mix for 30 minutes. Add an equal volume of Johns-Manville Hyflo-Supercel and mix for 30 minutes. Age for 24 hours.

Add 2 cm. of anhydrous granular sodium sulfate to the fritted-glass disk of the column. Add 6 cm. of adsorbant and top with 2 cm. of sodium sulfate. Pack with slight suction and with tamping. Prewash the column with 100 ml. of petroleum ether. Add the extract to the column and collect

the filtrate. Add four 25-ml. portions of petroleum ether and concentrate the filtrate to 5 ml.

Alfalfa, clover, timothy, and mixtures of these, or crops containing plant wax and fats. Prepare two adsorbants: For the first, mix equal volumes of Celite 545 with Attapulgas clay. For the second, follow the procedure under "Lettuce, green beans, peppers, potatoes, celery, tomatoes," starting at "As an adsorbant—" and ending with "Age for 24 hours," substituting Celite 545 for Hyflo-Supercel.

Air-dry the crop. Mill representative samples and blend. To a 300-gram sample, add 1800 ml. of *n*-hexane and extract by tumbling for 45 minutes. Allow the solids to settle, and filter the supernatant liquid through cotton gauze. Dry with sodium sulfate and add 10 grams of the first adsorbant. Swirl and filter, collecting 900 ml. Evaporate the *n*-hexane at 100°, removing the last trace of solvent with a gentle air current. Add 25 ml. of petroleum ether and reflux for 15 minutes.

Assemble a column, the same as described for the previous sample. Surround with a circulatory jacket, 38 × 220 mm., surrounding it from a point 20 mm. above the fritted-glass disk.

Prepare the refrigerated chromatographic column with 2 cm. of sodium sulfate, 3 cm. of the first adsorbant, 3 cm. of the second adsorbant, and 2 cm. of sodium sulfate. Level and pack each layer. Chill the methanol to be circulated through the jacket to -10° in a methylene dichloride dry-ice bath. Chill the column and prewash with 100 ml. of petroleum ether. Allow the petroleum ether to cool before drawing it through the column with slight suction. Discard the petroleum ether filtrate. Add 2 grams of Celite 545 to the extract, swirl, and add to the column. Let it cool for several minutes to permit the plant waxes and fats to solidify on the Celite. Add four 25-ml. portions of petroleum ether after each preceding portion is drawn through the upper sodium sulfate layer. Remove the suction after each volume is drawn into the column and allow the next volume to chill before drawing it through the column. Concentrate the filtrate to 5 ml.

Cottonseed and samples with high oil content. Grind delinted cottonseed in a Mikro-Sampl-Mill through a 1/16-inch screen. Blend a 100-gram sample for 5 minutes with 200 ml. of petroleum ether which has been redistilled from sulfuric acid. Filter through an asbestos mat and rinse quantitatively. Add 50 ml. of concentrated sulfuric acid to the filtrate and rotate. After 15 minutes, drop the acid-sludge through two

100-ml. portions of petroleum ether. Allow another 15-minute separation, and decant the petroleum ether washes and extracts. Wash the petroleum ether extract with 50-ml. portions of water, follow with 2% sodium bicarbonate solution, and repeat until the water wash is neutral. Dry the extracts with sodium sulfate. Concentrate the extract to 5 ml. Check the pH, as more potassium hydroxide may be required if sulfuric acid remains.

Procedure—Slurry Celite 545 with 1:1 hydrochloric acid, filter, and wash with water. Dry at 100°. To prepare the 1-naphthylamine reagent, dissolve 0.35 gram of 1-naphthylamine in 99 ml. of glacial acetic acid and 200 ml. of water. Add 7.5 grams of procaine hydrochloride and dilute to 1 liter with water.

To a 5-ml. sample solution containing 0.01-0.05 mg. of pentachloronitrobenzene, add 2 ml. of 2.8% potassium hydroxide solution in ethanol and 1 ml. of acetone. Heat for 7 minutes at 80° with the bottom of the flask immersed 1/4 to 1/2 inch in the water bath. Cool in cold water. Add 20 ml. of the reagent and adjust the pH to 2 to 2.5 with concentrated hydrochloric acid. If the sample had a high wax content, it is necessary to warm the solution at 80° for 3 minutes to free all nitrite from the waxes. Add 25 ml. of petroleum ether, stopper, and shake for 15 minutes. If the magenta color appears at the interface after shaking, add 1 ml. of butanol. Separate the layers and pass the aqueous layer through 1 × 1-cm. treated Celite in a medium porosity filter stick. Read at 525 m μ .

4-ETHYL-1-NITROBENZENE

4-Ethyl-1-nitrobenzene in air is absorbed in water, reduced to the amine with sodium hydrosulfite, diazotized, and coupled.⁴³ It will detect 2.5 micrograms per liter of air.

Procedure—Pass 10 liters of air during a half-hour through two gas-washing bottles, each containing 10 ml. of water. Combine the washings and take a 10-ml. aliquot. Add 0.2 ml. of a 2.5% solution of sodium hydrosulfite in 0.4% sodium hydroxide solution, and mix. After 5 minutes, add 2 ml. of 1:10 hydrochloric acid and 1 ml. of 3.5% aqueous sodium nitrite. Mix, and after 10 minutes add 3 ml. of 10% sodium carbonate solution. Add 2 drops of 2% aqueous solution of disodium-3-hydroxynaphthalene-2,7-disulfonate (R-salt). After 10 minutes, read at 500 m μ .

⁴³ T. Czajkowska and J. Piotrowski, *Chem. Anal., Warsaw* **8**, 917-24 (1963).

NITROBENZOIC ACIDS

The three isomers of nitrobenzoic acid are reduced to amines, separated by paper chromatography, and developed with *N*-(1-naphthyl)-ethylenediamine.⁴⁴

Procedure—To 10 ml. of solution containing about 0.01 gram of the mixed isomers, add 9 ml. of 1:8 hydrochloric acid and 4 ml. of 15% titanous chloride solution. After mixing and letting stand for an hour at room temperature, dilute it to 25 ml. Chromatograph on paper. The eluant is 35:35:17.5:12.5 methanol-benzene-amyl alcohol-water, pre-treated with sodium ethylenediamine tetraacetate.

Segregate the portions of the paper containing the isomers and suspend each in 8 ml. of 1:50 hydrochloric acid. Filter after extraction is complete. To 4 ml. of filtrate, add 2 ml. of 0.1% sodium nitrite solution. After 10 minutes at room temperature, add 1 ml. of 1% ammonium sulfamate solution. Stir, and add 1 ml. of 0.2% *N*-(1-naphthyl)ethylenediamine solution. Store without evaporation for 2 hours at 38°. Read at 550 m μ .

2-AMINO-5-NITROTHIAZOLE

2-Amino-5-nitrothiazole is diazotized in sulfuric acid and the normal diazo salt is coupled with *N*-naphthylethylenediamine.⁴⁵ The method is also applicable to the phthalyl and succinyl derivatives after hydrolysis. Blood samples must be deproteinized.

Procedure—*Blood*. Dilute a 1-ml. sample with 2 ml. of water. Add 1 ml. of 10% trichloroacetic acid solution. Mix well and centrifuge for 2 minutes. To 1-ml. of deproteinized sample containing up to 0.5 mg. of 2-amino-5-nitrothiazole in acid solution, add 5 ml. of 5:4 sulfuric acid, and cool in ice water for 2 minutes. Add 1 ml. of 2% sodium nitrite solution, mix, and let stand for 4 minutes. Add 1 ml. of 2.5% sulfamic acid solution, mix, and let stand for 4 minutes. Add 1 ml. of 0.4% *N*-naphthylethylenediamine hydrochloride solution. Mix, and let stand for 2 minutes. Remove from the ice bath, dilute to 25 ml. with 95% ethanol, and read within 5 minutes at 580 m μ .

⁴⁴ G. Coppi, *Boll. Chim. Farm.* **101**, 225-30 (1962).

⁴⁵ C. W. Ballard and S. Spice, *J. Pharm. and Pharmacol.* **4**, 1067-8 (1952).

SUCCINYLAMINONITROTHIAZOLE

After hydrolysis, succinylaminonitrothiazole is diazotized and the diazo salt is coupled with *N*-naphthylethylenediamine.⁴⁶

Procedure—To a 1-ml. solution containing up to 0.8 mg. of succinylaminonitrothiazole, add 5 ml. of 5:4 sulfuric acid, and heat at 100° for 40 minutes. Cool in ice water for 5 minutes and follow the procedure for 2-amino-5-nitrothiazole, starting at “Add 1 ml. of 2% sodium nitrite solution. . . .”

PHTHALYLAMINONITROTHIAZOLE

After hydrolysis, phthalylaminonitrothiazole is diazotized and the diazo salt is coupled with *N*-naphthylethylenediamine.⁴⁷

Procedure—Follow the procedure for succinylaminonitrothiazole, but heat for 90 minutes in place of 40 minutes.

1,2-DIMETHYL-5-NITROIMIDAZOLE

After reduction to 5-amino-1,2-dimethylimidazole, 1,2-dimethyl-5-nitroimidazole is developed by diazotizing and coupling with *N*-1-naphthylethylenediamine.⁴⁸ The procedure is applicable to 0.02-0.2 mg. per ml.

Procedure—Homogenize the sample and deproteinize with trifluoroacetic acid. Centrifuge. Dilute a known amount of centrifugate to 5 ml. with water. Add 5 ml. of 2:3 hydrochloric acid and 0.2-0.25 gram of zinc dust to reduce the imidazole to 5-amino-1,2-dimethylimidazole. After 15 minutes, dilute a portion of the solution equivalent to 0.02-0.2 mg. of 1,2-dimethyl-5-nitroimidazole to 2 ml. with 2:3 hydrochloric acid. Follow the procedure under 5-amino-1,2-dimethylimidazole (p. 505), starting with “Add 1 ml. of 1% sodium nitrite solution”

DICHLORAN, 2,6-DICHLORO-4-NITROANILINE

2,6-Dichloro-4-nitroaniline is extracted with benzene, purified by calcium chromatography on Florasil, evaporated to dryness, and taken up

⁴⁶ Loc. cit.

⁴⁷ Loc. cit.

⁴⁸ L. B. Colvin, R. Silvaramakrishnan and J. R. Couch, *Chemist Analyst* 52, 9-11 (1963).

in acetone. Addition of strong alkali produces an intense yellow color characteristic of some mononitro aromatic compounds in the presence of strong alkali and acetone.⁴⁹ Reduction of 2,6-dichloro-4-nitroaniline with zinc and hydrochloric acid to 2,6-dichloro-*p*-phenylenediamine followed by oxidation, in the presence of aniline, produces a blue indamine dye.⁵⁰ The temperature of the solution during oxidation must be below 25° and the concentration of hydrochloric acid must be very low. Acetic acid stabilizes the indamine dye and intensifies the color.

p-Nitroaniline, 2-chloro-4-nitroaniline, and 2,5-dichloro-4-nitroaniline give strong blue colors with the reagents. *o*-Nitroaniline, 4-chloro-2-nitroaniline, 2,4-dichloro-6-nitroaniline, 2,4-dinitroaniline, and 6-chloro-2,4-dinitroaniline give orange or brown colors. *m*-Nitroaniline, 2,4,5-trichloro-3-nitroaniline, 2,6-dichloro-4-nitroacetanilide, trichloraniline, and chloranil do not react. The following pesticides do not interfere: DDT, γ -BHC, derris, pyrethrum, malathion, Chlorparacide, colloidal sulfur, copper oxychloride, phenylmercuric nitrate, Melprex, TCNB, and PCNB. Less than 0.002 mg. of 2,6-dichloro-4-nitroaniline can be detected.

An alternative is to isolate from interferences, diazotize, and couple with *N*-1-naphthylethylenediamine for reading.⁵¹

Procedure—*By acetone and alkali. Canned and frozen peaches and apricots.* Open the canned fruit and pour over a 1/8-inch screen, draining for exactly 2 minutes. Collect the syrup. From this point on, the fruit and syrup are separate samples. Thaw the frozen fruit at 5° for 12 hours before opening. Chop the drained fruit into small pieces and place a 400-gram sample in a 1-gallon tin can with a metal baffle for mixing. Add 800 ml. of benzene. Seal tightly and roll on a mechanical roller for 30 minutes at 35 rpm. Decant the supernatant fluid into approximately 100 grams of anhydrous sodium sulfate and mix thoroughly. Filter through fluted filter paper and store in a tightly capped bottle.

Dilute 25-50 grams of syrup tenfold with water and extract with 100 ml. of diethyl ether for 1 minute. Let stand for 10-15 minutes and drain off the lower aqueous layer. Add the upper layer to 20 grams of anhydrous sodium sulfate. Filter, and store for analysis in a tightly stoppered bottle.

As a chromatographic column, add 25 grams of 60-100 mesh Florasil

⁴⁹ C. C. Porter, *Anal. Chem.* **27**, 805-7 (1955); Wendell W. Kilgore, Kim Wa Cheng and Joseph M. Ogawa, *J. Agr. Food Chem.* **10**, 399-401 (1962).

⁵⁰ J. Roburn, *J. Sci. Food Agric.* **12**, 766-72 (1961).

⁵¹ H. J. Hendon, H. Brummik and E. W. van der Pol, *Dtsch. Lebensmitt. Rorsch.* **60**, 67-8 (1964).

to a 2.5×25 -cm. column. This should not be reactivated. Wet it with benzene. Pass 100 ml. of either extractive through the column, discarding the first 50 ml. of effluent. Wash as usual with 100 ml. and 100 ml. of 1% ether in benzene. Evaporate the solvent from the eluate *in vacuo* at 60° . One minute after the last solvent disappears, blow out benzene vapor with a stream of air. Any residual solvent will produce turbidity later.

Rinse down the sides of the flask with 3 ml. of acetone and filter through glass wool. Rinse the flask with one 2-ml. and one 1-ml. portion of acetone, filtering each rinsing. Dilute the combined filtrates containing 0.0025-0.02 mg. of dichloran to 5 ml. with acetone and add 0.15 ml. of 4% potassium hydroxide solution. Read the yellow color against a reagent blank at $464\text{ m}\mu$ within 30 minutes.

By reduction followed by oxidation in the presence of aniline. Lettuce. Surface deposits. Weigh the sample and separate the leaves. Add 4 ml. of toluene per gram of sample. Stopper, and shake mechanically for 15 minutes. Filter the toluene and take an aliquot of 3-40 ml. containing 0.03-0.15 mg. of 2,6-dichloro-4-nitroaniline.

To the toluene extract, add 0.5 gram of zinc, 15 ml. of water, and 0.5 ml. of 1:10 hydrochloric acid. Fit the flask with a solvent trap with its tap closed and an air condenser 50-70 cm. in length. Details of the trap are shown in Figure 1. Shield the apparatus from strong light and

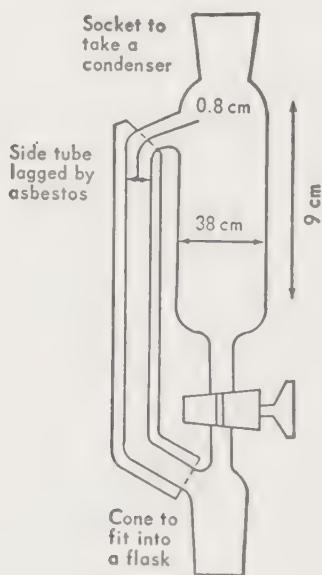


FIG. 1.

Solvent trap for determination of 2,6-dichloro-4-nitroaniline

heat. When the toluene starts to steam distil into the trap, let down the lower layer periodically into the flask so that the contents of the flask are always above 7 ml. After all the toluene is distilled off, continue refluxing for 15 minutes. When the boiling stops, allow the water in the trap to run into the flask. Cool in the dark to 25° or below. Filter if necessary.

To a 10-ml. portion, add 2 ml. of 0.2% aniline solution in glacial acetic acid and 1 ml. of 0.05% potassium dichromate solution. Read at 650 m μ 12 minutes after addition of the dichromate.

Lettuce. Total residue. Cut a 100-gram sample into strips 0.5-1 inch wide. Macerate with 200 ml. of acetone for 3 minutes and shake on a mechanical shaker for 30 minutes. Filter with suction and wash with 50 ml. of a 3:1 acetone-water mixture. Measure the volume of the filtrate. To 20 ml. of the filtrate, add 20 ml. of toluene and shake. Extract the toluene layer with 2 ml. of 4% sodium hydroxide solution. Discard the aqueous layer. Repeat the extractions with two 10-ml. portions of 0.8% sodium hydroxide solution and one 10-ml. portion of 1:24 hydrochloric acid, discarding all aqueous layers. Pass the extract through a dry 5 \times 2-cm. alumina column and elute with 40 ml. of 1:9 acetone in toluene. Collect the entire eluate.

Take an aliquot of the eluate containing 0.01-0.2 mg. of 2,6-dichloro-4-nitroaniline. Develop as for surface deposits, starting at "To the toluene extract add 0.5 gram of zinc . . ."

Fruit. Surface deposits. Shake the sample for 15 minutes with 1 ml. of toluene for each gram of fruit. Take an aliquot containing 0.02-0.15 mg. of 2,6-dichloro-4-nitroaniline. If the blank with untreated fruit is higher than a reading corresponding to approximately 0.001 mg. of 2,6-dichloro-4-nitroaniline, pass the aliquot of the sample through a 3 \times 2-cm. dry alumina column and elute with 40 ml. of 1:9 acetone in toluene. Collect the entire eluate and develop as for surface deposits on lettuce, starting at "To the toluene extract add 0.5 gram of zinc . . ."

Fruit. Total residue. If the fruit is larger than 0.5 inch in diameter, cut into small pieces. Follow the procedure under "Lettuce. Total Residue."

Sol. Put a weighed sample containing not more than 0.5 mg. of 2,6-dichloro-4-nitroaniline in a filter paper thimble and extract with acetone

for 1 hour in a Soxhlet apparatus. Dilute the acetone extract to 50 ml. and add 50 ml. of toluene. Wash with three 100-ml. portions of 1% sodium sulfate solution. Mix 20 ml. of the extract with 2 ml. of acetone and pass through a 2×2 -cm. alumina column. Elute with 30 ml. of a 1:7 mixture of acetone and toluene. Develop as for surface deposits on lettuce, starting at "To the toluene extract add 0.5 gram of zinc. . . ."

By N-1-naphthylethylenediamine. Fruit. Extract 100 grams of the juice or of a homogenized sample with 200 ml. of ether for 30 minutes. Acidify an aliquot of the extract and slowly evaporate the solvent. Add 5 ml. of water, 10 grams of magnesium sulfate, and 2 ml. of 16% sodium hydroxide solution to the residue. Steam distil into 10 ml. of 1:35 sulfuric acid. Make the steam distillate alkaline with 16% sodium hydroxide solution and extract with 10 ml. and 5 ml. of dichloromethane.

Dry the combined extracts with anhydrous sodium sulfate. Filter, acidify with acetic acid, and evaporate the dichloromethane. Chill the residue to zero and diazotize with 1 ml. of nitrosyl sulfate. After 10 minutes, add 1 ml. of a 15% solution of ammonium sulfamate. After 10 minutes, add 1 ml. of 1% *N*-1-naphthylethylenediamine solution. Dilute to 10 ml. with water. After 15 minutes read at 505 $m\mu$.

5,2'-DICHLORO-4'-NITROSALICYLICANILIDE

This molluscicide is necessarily determined in microgram amounts in water. It may be extracted from acid solution with chloroform and developed with ethanolamine.⁵² An alternative is to complex with safranin-O in mildly alkaline solution and extract with chloroform. Modifications of the safranin procedure call for extraction with 9:1 amyl acetate-hexane.⁵³

Procedure—Water. Acidify a 200-ml. sample with 10 ml. of 1:17 sulfuric acid. Shake vigorously with 10 ml. of chloroform for 30 seconds and let separate for 5 minutes. Separate the chloroform layer, add 2 ml. of ethanolamine, and shake. Read within 30 minutes at 385 $m\mu$.

Alternatively, add 5 ml. of saturated sodium bicarbonate solution to 100 ml. of water. Add 5 ml. of 0.02% safranin-O and 10 ml. of chloroform. Shake for 30 seconds and let separate for 5 minutes. Read the chloroform layer at 515 $m\mu$.

⁵² Keith J. Farrington, *Anal. Chem.* **34**, 1338-9 (1962).

⁵³ Reimer Strufe, *Bull. World Health Organ.* **25**, 503-7 (1961).

4-NITROPHENYLARSONIC ACID

After reduction of the 4-nitrophenylarsonic acid with hydrosulfite, the color is developed by coupling with *N*-(1-naphthyl)ethylenediamine dihydrochloride.⁵⁴ Beer's law is followed up to 0.5 mg. per 100 ml. 3-Nitro-4-hydroxyphenylarsonic acid does not interfere. Compounds with an amino or reducible nitro group do interfere. A method by silver diethyl-dithiocarbamate is less satisfactory.⁵⁵

Procedure—Feeds. Heat a 6-gram sample with 80 ml. of methanol for 20 minutes at 100°, shaking frequently. Cool, and dilute to 100 ml. with methanol. Mix, and let stand for 2 hours. Evaporate a 25-ml. portion of the solution almost to dryness at 100°. Add 50 ml. of 1% sodium hydrosulfite solution in 4% sodium hydroxide solution. Heat at 100° for 30 minutes. While hot, add concentrated hydrochloric acid until a permanent turbidity remains on shaking, adding 1 ml. of acid in excess. Aerate with air or nitrogen at the rate of 1 liter per minute for 20 minutes. Cool, dilute to 100 ml. with water, and filter, discarding the first 5 ml. of filtrate.

Prepare two 4-ml. aliquots. To one, add 1 ml. of 0.5% sodium nitrite solution. Mix by inverting and let stand for 5 minutes. Add 1 ml. of 2.5% ammonium sulfamate solution, and after 2 minutes, add 2 ml. of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride solution, and mix. Treat the second 4-ml. aliquot in the same way, using 2 ml. of water in place of the coupling reagent. After 10 minutes, read at 540 m μ , using the second sample as the blank.

3-NITRO-4-HYDROXYBENZENE-ARSONIC ACID

3-Nitro-4-hydroxybenzenearsonic acid gives an intense yellow color in alkaline solution.⁵⁶ Proteins are precipitated from a solution that has been adjusted to the isoelectric point of the protein by hydrochloric acid. Interfering substances including acetyl-(*p*-nitrophenyl)sulfanilamide, nicarbazin, Enheptin, furazolidone, nitrofurazone, and Megasul are adsorbed on activated charcoal at pH 12. 4-Nitrophenylarsonic acid and diphenyl-*p*-phenylenediamine do not interfere. Beer's law is followed for 0.1-2.2 mg. per 100 ml.

⁵⁴ J. W. Cavett, *J. Assoc. Offic. Agr. Chemists* **39**, 967-9 (1956).

⁵⁵ H. H. Hoffman, *ibid.* **46**, 456-9 (1963).

⁵⁶ J. W. Cavett, *ibid.* **39**, 857-60 (1956).

Procedure—Feeds. To a 5-gram sample, add 50 ml. of 2% sodium bicarbonate solution, shake to mix, and let stand for 15 minutes. Centrifuge for 5 minutes at 2000 rpm. Decant 30 ml. of the centrifugate through a layer of cheesecloth into a 40 ml. centrifuge tube. Stand the tube upright with a strong light behind it. Place a stirring rod in the tube and carefully add 8-10 drops of concentrated hydrochloric acid, stirring until the effervescence disappears. Adjust the pH to the isoelectric point of the proteins, which is 4-5, by dropwise addition of concentrated or 1:1 hydrochloric acid. Stir. Allow enough time between drops to see whether the proteins flocculate. Use wide-range pH paper to help determine this point. After the proteins have flocculated, let the solution stand a few minutes and centrifuge for 10 minutes at 2500 rpm.

Decant through a single layer of cheesecloth. The solution should have a pH of 4-5 and should be a clear yellow. Add 4 drops of 50% sodium hydroxide solution to bring the pH to 12. Add 2 grams of activated charcoal, let stand for 30 minutes, shaking occasionally, and filter. Repeat the process with an 0.5 gram portion of activated charcoal.

To a portion of the filtrate, add 1 drop of concentrated hydrochloric acid. Read at 410 $m\mu$ against water. Read another portion without addition of acid. The difference represents 3-nitro-4-hydroxybenzenearsonic acid.

Premixes. To a 5-gram sample, add 200 ml. of 2% sodium bicarbonate solution. Let stand for 20 minutes, shaking occasionally, and let settle for 30 minutes. For premixes containing more than 0.5% of 3-nitro-4-hydroxybenzenearsonic acid, dilute a 5-ml. aliquot to 100 ml. with water. If less than 0.5% is present, use a 10-ml. aliquot. If there is much turbidity, add 1 gram of Celite, shake, and centrifuge. Adjust the pH of 30 ml. of the supernatant liquid to 3 with 1 drop of hydrochloric acid, and shake to remove carbon dioxide. Add 4 drops of concentrated sodium hydroxide solution and 2 grams of activated charcoal. Let stand for 30 minutes, shaking occasionally. Repeat the charcoal treatment with 0.5 gram of charcoal. Follow the procedure under "Feeds," starting at "To a portion of the filtrate, add 1 drop . . ."

PHOSPHACOL, DIETHYL *p*-NITROPHENYL PHOSPHATE

Diethyl *p*-nitrophenyl phosphate is determined by sodium perborate and *o*-dianisidine solution in acetone.⁵⁷ The sensitivity is 0.2 mg. in 100 ml.

⁵⁷ A. F. Fursov, *Aptekhn. Delo* 11, 46-9 (1962).

Procedure—To a sample containing 0.002-0.045 mg. of diethyl *p*-nitrophenyl phosphate, add 1 ml. of sodium perborate solution and 2 ml. of 1% *o*-dianisidine solution in acetone. Dilute to 10 ml. with ethanol and read at 460 $m\mu$ after 25 minutes.

NITROFURAMINE, 5-NITRO-2-FURFURYLIDENEHYDRAZIDE
OF ISONICOTINIC ACID

Nitrofuramine is read in alkaline solution or extracted and read as the hydrazone.⁵⁸

Procedure—*Direct reading.* Extract pharmaceutical preparations with acetone in a Soxhlet unit. Dilute an appropriate aliquot of the extract, such as 0.2 ml. to 10 ml., with 1:1 acetone-water. Add 2 ml. of 10% sodium hydroxide solution in 50% ethanol and read at 560 $m\mu$.

As the hydrazone. To 0.1 ml. of acetone extract of nitrofuramine, add 2 ml. of 1.5% aqueous phenylhydrazine hydrochloride. Digest at 70° for 25 minutes and cool. Extract the hydrazone with 10 ml. of toluene and read at 430 $m\mu$.

NITROFURAZONE, 5-NITRO-2-FURALDEHYDE SEMICARBAZONE

The determination of nitrofurazone is based on the formation and the photometric estimation of the red 5-nitro-2-furfuraldehyde phenylhydrazone.⁵⁹ The details as applied to feeds are given in Volume IIIA, pages 270-2. Further work on biological samples is given here.

The phenylhydrazone is extracted with toluene. In liver samples, the phenylhydrazone is separated by chromatography on aluminum oxide from nonspecific liver chromogens.⁶⁰ Nitrofurazone in concentrations as low as 0.5 ppm. can be determined after separation. The range of accuracy is 1 to 5 ppm. A technique that follows for furmethanol in milk is also applicable to nitrofurazone. Dioxan may replace toluene as the extraction

⁵⁸ M. Marti, I. Bulto, T. Vilarroya, and J. Vares, *Galenica Acta* (Madrid) **10**, 111-17 (1957).

⁵⁹ James A. Buzard, Dorothy M. Vrablic and Mary F. Paul, *Antibiotics and Chemotherapy* **6**, 702-7 (1956); James A. Buzard, V. R. Ellis and M. F. Paul, *J. Assoc. Offic. Agr. Chemists* **39**, 512-8 (1956); Haruo Ikeda, *J. Food Hyg. Soc. Japan* **3**, 269-72 (1962).

⁶⁰ Ronald J. Herrett and James A. Buzard, *Anal. Chem.* **32**, 1676-8 (1960).

medium,⁶¹ with the extract read directly at 450 m μ . In liver samples, metaphosphoric acid is added to inactivate the liver. Since nitrofurazone is affected by metaphosphoric acid, the sample and acid are kept cold.

In plasma or serum samples, Beer's law is followed in the limited range of 0.01-0.03 mg. per 3-ml. sample. The following, which may be found in plasma or serum, do not interfere: chlortetracycline, oxytetracycline, chloramphenicol, streptomycin, dihydrostreptomycin, penicillin, bacitracin, enheptin, nicarbazine, sulfaquinoxaline, nitrophenide, arsanilic acid, 3-nitro-4-hydroxyphenylarsonic acid, *p*-aminobenzoic acid, thiouracil, and phenothiazine. Since the conversions of nitrofurantoin and furazolidone to the corresponding phenylhydrazones is incomplete, it is necessary to run standard solutions of the nitrofuran with each analysis.

Another method for nitrofurazone consists of reading in isopropanol at 375 m μ , followed by reduction with hydrosulfite. The residual color after reduction is the correction for background interference.⁶² In water, the maxima are at 260 m μ and 375 m μ ; in ethanol, at 262 m μ and 365 m μ ,⁶³ in dilute diethyleneglycol at 373 m μ .⁶⁴

Nitrofurazone is developed with phenol in alkaline dimethylformamide and read against a similar solution reduced with hydrosulfite.⁶⁵ Nitrofurazone in milk is determinable down to 1 ppm. by precipitation of proteins by sodium tungstate and sulfuric acid and by direct reading at 375 m μ .⁶⁶

Procedure—*By phenylhydrazine. Plasma or serum.* To a 3-ml. oxalated, citrated, or heparinized sample, add 5-10 mg. of sodium hydrosulfite, and shake occasionally for 15 minutes to destroy any nitrofuran present. Use this tube as a control. To both the control and another 3-ml. sample, add 1 ml. of 1.5% phenylhydrazine solution and 1 ml. of 1:1.5 hydrochloric acid. Shake, and heat for 25 minutes at 70°. Cool in cold water for 5 minutes and add 5 ml. of toluene. Shake vigorously for approximately 25 times and centrifuge. Break the curd-like top layer and recentrifuge the toluene layer if it is not clear. Read the toluene layer at 430 m μ against toluene. Subtract the absorbance of the control from that of the sample.

⁶¹ M. Sanz Munoz, *An. Acad. Farm. Madrid* **27**, 289-302 (1961).

⁶² W. R. Flach, *J. Assoc. Offic. Agr. Chemists* **39**, 318-21 (1956).

⁶³ S. Radnic and M. Maksimovic, *Archiv. Farm.* (Belgrade) **12**, 249-55 (1962).

⁶⁴ W. Zyzynski, *Acta Polon. Pharm.* **18**, 365-70 (1961).

⁶⁵ The Additives in Animal Feeding-stuffs Committee of the Society for Analytical Chemistry, *Analyst* **88**, 935-40 (1963).

⁶⁶ G. E. Parr, *J. Agr. Food Chem.* **10**, 291-2 (1962).

Chicken liver. Samples are frozen in powdered dry ice and stored until analysis. Homogenize a 5-gram portion of the thawed cold sample with 8 ml. of cold 5% metaphosphoric acid solution. Take up with 3 ml. of cold 5% metaphosphoric acid solution and cold water, and dilute to 30 ml. with cold water. Add 3 ml. of 1.5% phenylhydrazine hydrochloride solution. Heat at 70° for 10 minutes and cool. Add 3 ml. of concentrated hydrochloric acid, shake to mix, and heat for 25 minutes at 70°. Cool, shake for 1 minute with 10 ml. of toluene, and centrifuge. Take 8 ml. of the toluene layer for chromatography on an aluminum oxide column.

To prepare the chromatographic column, pack a 25 × 1-cm. column with dry anhydrous aluminum oxide to a height of 6 to 7 cm. with a glass wool plug at the bottom. Wash with toluene and add a glass wool plug at the top. Equilibrate the column with toluene. Add the 8-ml. sample, followed by 10 ml. of toluene. Add a 3:1 toluene-ethyl acetate mixture, which separates the colored material into four bands: a yellow liver material at the bottom, the red phenylhydrazone derivative next, a second yellow liver derivative, and a stationary brown band. The toluene-ethyl acetate mixture elutes the first yellow liver zone.

After the first liver band is eluted, add enough 1:1 toluene-ethyl acetate mixture to move the red phenylhydrazone band to the bottom of the column. Elute this band with 10-15 ml. of pure ethyl acetate. Concentrate this eluate to less than 0.5 ml. with a flash evaporator for 15 minutes at room temperature on a water aspirator pump. Dilute to 5 ml. with toluene and read at 400-460 $m\mu$ against a toluene blank. Subtract the absorbance of an untreated liver sample.

Fat and muscle tissue. Mince 5 grams of cold tissue and add to 10 ml. of 50% N,N-dimethylformamide at 90-100°. Heat at 100° for 30 minutes and dilute to 15 ml. with water. Extract the fat from the sample with 12 ml. of toluene, and discard the toluene. Add 10 ml. of the remaining extract to a mixture of 1 ml. of 1.5% phenylhydrazine hydrochloride solution and 1 ml. of concentrated hydrochloric acid. Heat at 70° for 25 minutes and cool. Extract with 5 ml. of toluene. Read the toluene layer at 400-460 $m\mu$ against toluene.

Direct reading. Extract 9.0 grams of 20-mesh sample with hexane. Air-dry the residue and extract with 100 ml. of isopropanol. Filter an aliquot of the isopropanol extract and add water to dilute to an appropriate absorption level. Read at 375 $m\mu$. Add a crystal of sodium hydrosulfite

to the cuvet and read again after 20 minutes. Subtract the second reading from the first.

Alternatively, extract about 100 mg. of nitrofurazone or furazolidone with 100 ml. of diethylene glycol. Dilute 5 ml. with 95 ml. of a buffer for pH 10. Read nitrofurazone at 373 $m\mu$. Read furazolidone at 365 $m\mu$.

Feedstuffs. Extract a sample expected to contain about 1 mg. of nitrofurazone with petroleum ether. Discard the extract. Evaporate the solvent from the thimble at not over 60° in a current of air. Extract with acetone and thereafter protect this from light. Cool the acetone extract and add about 4 drops of 0.32% potassium permanganate solution. If the color does not persist for more than 2 seconds, add more permanganate. Evaporate to about 5 ml., and then evaporate to dryness with a stream of air at not over 60°. Take up the residue in dimethylformamide and dilute to 50 ml. with that solvent. Add portions equivalent to about 0.3 mg. of nitrofurazone to each of two flasks. Add 5 ml. of 5% phenol in dimethylformamide to each. To the sample flask, add 2.5 ml. of 4% sodium hydroxide solution, and dilute to 50 ml. with dimethylformamide. To the other flask, which is to be the blank, add 2.5 ml. of fresh 1% solution of sodium hydrosulfite in 4% aqueous sodium hydroxide. Dilute to 50 ml. with dimethylformamide. The blank should be yellow. Centrifuge, and read the sample against the blank at 530 $m\mu$.

*Milk.*⁶⁷ For milk containing under 0.1 ppm. of nitrofurazone, transfer 30 ml. to each of four 40-ml. centrifuge tubes. Add 0.5 ml. of 1% phenylhydrazine hydrochloride and 2 ml. of concentrated hydrochloric acid to each. Shake, and heat at 70° for exactly 15 minutes. Chill in ice and water for 5 minutes. Add 8 ml. of toluene and approximately 6 grams of salt to each. Shake vigorously for 1 minute and centrifuge for 10 minutes. Decant and pool the toluene layers, 25-29 ml.

Set up a 300 × 10-mm. chromatograph tube. Add a plug of glass wool and pour in a slurry of 27 grams of alumina in 10 ml. of toluene. Wash down with more toluene and top with a pledget of glass wool. Add 25 ml. of the sample in toluene. When the last of this enters the column, add 15 ml. of toluene. The column will show a red band of the nitrofurane phenylhydrazone at the top of the column just below a yellow band of milk protein. When the toluene has drained, add exactly 2 ml. of 10:1 toluene-isopropanol. When this has passed into the adsorbent, the band should be midway of the column. Add 5 ml. of the toluene-isopropanol

⁶⁷ Loyal R. Stone, *J. Agr. Food Chem.* **12**, 121-3 (1964).

and collect 5 ml. of eluate. Read at 530 $m\mu$ as a blank. Add 1 drop of a molar solution of Hyamine hydroxide in methanol. Read the developed color at 530 $m\mu$. This reaction as the hydrazone is reproducible but not complete. Therefore, standards must be prepared with milk.

FURAZOLIDONE, 3-(5-NITRO-FURFURYLIDENEAMINO)-2-OXAZOLIDINE

Furazolidone is extracted from feed with hot dimethylformamide. This solution diluted 50:50 with carbon tetrachloride is applied to an alumina chromatographic column. Interfering colors are washed through with carbon tetrachloride. The furazolidone is eluted with isopropanol and carbon tetrachloride. Thereafter, furazolidone is extracted from the eluate with water and read at 365 $m\mu$.⁶⁸ On a standard column, the furazolidone is a faint yellow band. On columns of feed samples, this is usually obscured by other colors. Moisture in the solvent affects the adsorption on alumina and the rate of elution from the column. A corresponding amount is added to the standard.

Furazolidone is estimated as the red phenylhydrazone derivative by the same methods that apply to nitrofurazone (p. 32). It is also determinable by reading in the ultraviolet.⁶⁹

Procedure—As a standard stock solution, dilute 55 mg. of furazolidone to 100 ml. with dimethylformamide. Protect from light. As a working standard, dilute 5 ml. of the stock solution with 45 ml. of dimethylformamide. Add 0.5 ml. of water and dilute to 100 ml. with carbon tetrachloride. Prepare the working standard daily.

The chromatographic column is 30 \times 8 mm. with a glass wool plug. Make a thin slurry of aluminum oxide, such as Merck 71707, in toluene. Build up a bed 9.5 \pm 0.5 cm. deep. Let the toluene drain to the bed level for use.

Mix 5 grams of feed with 10 ml. of dimethylformamide and, loosely stoppered, heat at 100 $^{\circ}$ for 5 minutes. Shake for 5 minutes while cooling and add 10 ml. of carbon tetrachloride. Filter. If the feed contains less than 0.02% of furazolidone, pipet 2 ml. onto the freshly prepared chromatographic column. If it contains more, use 1 ml. As the sample enters the bed, add 5 ml. of carbon tetrachloride. Let the column drain and discard the effluent.

⁶⁸ Lloyd R. Stone, *J. Assoc. Official Agr. Chemists* **47**, 562-5 (1964). Cf. J. Pasich and M. Lehmann, *Farmacia Pol*, **20**, 731-3 (1964).

⁶⁹ J. Uriach and R. Pujol, *Galencia Acta* (Madrid) **13**, 415-21 (1960).

Elute the furazolidone with 10 ml. of 1:1 carbon tetrachloride-isopropanol. Collect the effluent in a centrifuge tube. Add 5 ml. of water and shake vigorously. Centrifuge, and read the aqueous layer at 365 m μ . Also read the results of 2 ml. of working standard similarly treated, except that around 0.0055% of furazolidone, use 1 ml. of standard.

$$\% \text{ furazolidone in the feed} = (A_1/A_2) \times (V_2/V_1) \times 0.011$$

in which A_1 and A_2 are the respective absorbancies of the sample and standard, V_1 and V_2 their volumes placed on the column.

FURAZOLIDONE AND NITROFURAZONE

The commercial product Bifuran as present in feed is usually 0.0008% of furazolidone and 0.0056% of nitrofurazone. These can be separated in an alumina column for determination.⁷⁰ The method is derived from a method for determining the sum of the two.⁷¹

Procedure—As a nitrofurazone standard, dissolve 100 mg. in dimethylformamide and dilute to 100 ml. As a furazolidone standard, dissolve 100 mg. in dimethylformamide and dilute to 100 ml. Protect these from light. As a daily working standard, add 1.4 ml. of the nitrofurazone standard and 0.2 ml. of the furazolidone standard to 48 ml. of dimethylformamide. Add 1 ml. of water and dilute to 100 ml. with carbon tetrachloride.

Add 10 ml. of dimethylformamide to a 5-gram sample and stopper loosely. Agitate constantly at 70° for 10 minutes. Cool, add 10 ml. of carbon tetrachloride, and filter. Add 5 ml. of filtered extract to the alumina column (p. 35, furazolidone method) and similarly, 5 ml. of standard on another. When all the liquid enters the absorbent, add 5 ml. of carbon tetrachloride. Discard the eluate.

When the carbon tetrachloride enters the column, elute furazolidone with 8 ml. of 1:1 carbon tetrachloride-isopropanol. Collect the eluate in centrifuge tube A.

Now elute nitrofurazone from the column with 10 ml. of 10:1:1 chloroform-glacial acetic acid-ethanol, collecting the eluate in centrifuge tube B.

Add 5 ml. of water to each and shake vigorously. Centrifuge for 1

⁷⁰ Loyal R. Stone, *J. Assoc. Official Agr. Chemists* **47**, 565-7 (1964).

⁷¹ *Official Method of Analysis of the Association of Official Agricultural Chemists*, 9th Ed., p. 552 (1960).

minute. Transfer 5 ml. of water phase of each to tubes. To each, add 1 ml. of a freshly prepared 1:1 mixture of 1.5% phenylhydrazine hydrochloride solution and concentrated hydrochloric acid. Heat at 70° for 25 minutes and cool. Add 5 ml. of toluene to each and shake. Centrifuge and read the toluene layers at 440 $m\mu$.

5-NITRO-2-FURFURALDAZINE

For determining 5-nitro-2-furfuraldazine in nitrofurazone, it is extracted with chloroform. The need for the determination arises from its manufacture by the reaction of 5-nitrofurfuryl diacetate with semicarbazide hydrochloride.⁷² The test substance passes through an alumina chromatographic column in a purity suitable for direct reading.⁷³ Beer's law applies for 0.8-6 mg. per liter.

Procedure—Use a 35 × 1-cm. chromatographic tube, with the restricted end joined to a short 1-mm. capillary. Insert a cotton plug and build up a 5-6 cm. column of alumina, consolidated by tapping. Wash with 20 ml. of chloroform, draining the excess nearly to the surface of the alumina.

Mix 100 grams of nitrofurazone with 20 ml. of chloroform and warm on a steam bath for 5 minutes. Transfer the solution and as much solid as possible to the chromatographic tube. Allow to drain by gravity into a 100-ml. calibrated flask until almost to the surface of the alumina. Rinse the original container with 10, 10, and 10 ml. of chloroform, adding each to the column as before. Dilute to volume with chloroform and read at 378 $m\mu$ against chloroform. If too dark, further dilute an aliquot. If the sample contained 3% of the test substance, a further 10:1 dilution is appropriate. Protect all solutions from light. The value of $E_{1\text{cm}}^{1\%}$ is 1359 at 20°.

NITROFURFURYLACRYLAMIDE

After treatment with sodium hypochlorite, nitrofurfurylacrylamide is read in the visible range.⁷⁴ Nitrofurazone may be present and read at the same time.⁷⁵

⁷² Eaton Laboratories, British Patent 649,027 (1951).

⁷³ R. F. Bird and S. G. E. Stevens, *Analyst* **87**, 362-5 (1962).

⁷⁴ Haruo Ikeda, *J. Food Hyg. Soc. Japan*, **3**, 269-72 (1962).

⁷⁵ Saburo Kanno and Taeko Okimaya, *J. Food Hyg. Soc. Japan*, **4**, 198-204 (1963).

Procedure—To 10 ml. of sample containing about 5 ppm. of the acrylamide, with or without some nitrofurazone, add 0.25 ml. of sodium hypochlorite solution containing 5% of available chlorine. Hold at 50° for 10 minutes, cool, and read at 505 m μ for nitrofurazone. Add 0.5 ml. of 0.35% solution of phenylhydrazine in 1:3 hydrochloric acid. Keep at 75° for 25 minutes. Cool, and shake with 10 ml. of toluene. Read the extract at 525 m μ for the acrylamide.

NIHYDRAZONE, 5-NITRO-2-FURFURALDEHYDE ACETYLHYDRAZONE

After extraction from feed samples with dimethylformamide, nihydrazone is converted to the phenylhydrazone at 40°, extracted with toluene and read at 400 m μ .⁷⁶ Nitrofurazone drugs interfere. The dimethylformamide extract is passed through a magnesium hydroxide-aluminum oxide column to remove other interferences.

Sample—*Feed*. To prepare the absorbent, add 4 parts of magnesium hydroxide to 100 parts of aluminum oxide. Shake to mix. Add 5 parts of water and mix immediately to disperse lumps. Prepare a 5 \times 20-mm. adsorption column with the adsorbent. If the sample is highly colored, use a longer column. Place a glass wool or cotton plug at the bottom of the column, and top the adsorbent with washed sea sand. As a reagent, dissolve 0.5 gram of phenylhydrazone hydrochloride in 50 ml. of water and add 50 ml. of concentrated hydrochloric acid.

To a 10-gram sample, add 50 ml. of 95% dimethylformamide. Stopper loosely and heat at 100° for 5 minutes or until the temperature of the solution reaches 90°. Shake mechanically for 10 minutes and filter through rapid paper. Add 25 ml. of water to 25 ml. of the filtrate, and mix. Let stand in the dark for at least 30 minutes.

Pass the sample through the column and collect 15 ml. Pipet 5-ml. aliquots into each of two numbered tubes. Protect one tube from light. To the other tube, add 3 drops of freshly prepared 2% sodium hydro-sulfite solution. Mix, and let stand for 5 minutes. Treat 5-ml. aliquots of the diluted standard in the same manner. Pipet 5 ml. of the phenylhydrazone reagent into each of the tubes containing samples and standards. Mix, and heat at 40° for 20 minutes. Cool in water at about 15° for 5 minutes. Add 10 ml. of toluene to each tube, stopper, and shake

⁷⁶ Loyal R. Stone, *J. Assoc. Offic. Agr. Chemists* **44**, 2-4 (1961).

vigorously 40 times. Centrifuge to separate the toluene layer and read it at 400 m μ . Calculate as follows:

$$\frac{\text{absorbance}_{\text{sample}} - \text{absorbance}_{\text{reduced sample}}}{\text{absorbance}_{\text{standard}} - \text{absorbance}_{\text{reduced sample}}} \times 0.009 = \% \text{ nihydrazone}$$

**FURMETHANOL, FURALTADONE, N-(5-NITRO-2-FURFURYLIDENE)-
3-AMINO-5-N'-MORPHOLINYLMETHYL-2-OXAZOLIDONE**

The color developed by furmethanol with phenylhydrazine hydrochloride is applicable to the determination in milk. With a slight modification, it is also applicable to nitrofurazone.⁷⁷ The method is applicable to the range of 0.25-5 ppm. Furaltadone, after isolation from milk, is read directly.⁷⁸

Procedure—*By phenylhydrazine.* To 12 ml. of milk, add 4 ml. of 1.5% phenylhydrazine hydrochloride solution and 4 ml. of 1:1 hydrochloric acid. Heat in a water bath at 70° for 25 minutes for furaltadone or 15 minutes for nitrofurazone. Cool in ice and extract with 20 ml. of toluene. Centrifuge. Transfer 10 ml. of the toluene extract to a 6 × 1-cm. alumina column, previously washed with toluene. Wash the chromatographed extract through with 10 ml. of toluene. Follow this with 10 ml. of 3:1 toluene-ethyl acetate. Thereafter, wash with 1:1 toluene-ethyl acetate until the red hydroazone band approaches the bottom of the column. Elute with about 20 ml. of ethyl acetate. Evaporate the eluate to dryness and take up in 5 ml. of toluene. Read at 430 m μ .

Direct reading. Milk. Warm a 200-ml. sample to 40° and add 2 ml. of glacial acetic acid dropwise with stirring. When all the casein has flocculated, usually after 5 minutes of stirring, centrifuge for 30 minutes. Aspirate or decant 150 ml. through glass wool, avoiding fat or casein. Adjust the pH to 6.0 with 11.2% potassium hydroxide solution. Shake vigorously with 50 ml. of chloroform for 1 minute and centrifuge for 15 minutes. Break up the gel interface with a stirring rod and centrifuge for 30 minutes. Remove 35 ml. of uncontaminated chloroform phase with a syringe. Shake with 5 ml. of 1:100 hydrochloric acid for 1 minute and centrifuge for 10 minutes. Read the acid layer at 360 m μ against 1:100 hydrochloric acid. Correct with a determination on uncontaminated milk.

⁷⁷ Philip L. Cox and James P. Heotis, *J. Agric. Food Chem.* **10**, 402-3 (1962); R. J. M. Ratcliffe, *J. Assoc. Publ. Analysts* **1**, 78-85 (1963).

⁷⁸ Philip L. Cox and James P. Heotis, *J. Agric. Food Chem.* **11**, 499-501 (1963).

AROMATIC NITRATE INSECTICIDES

For determination of aromatic nitrate insecticides, they are reduced to amines and coupled with *N*-1-naphthalenediamine.⁷⁹ The method is applicable to micro amounts of Binapeeryl, EPN, parathion, and methyl parathion.

Procedure—To a sample containing up to 0.05 mg. of insecticide, add 0.5 ml. of 1% solution of lanolin in chloroform. Evaporate to dryness at 40° with the assistance of a stream of air. Add 5 ml. of a fresh mixture of equal parts of 95% ethanol and concentrated hydrochloric acid. Add 0.8 gram of zinc and reflux gently with an air condenser for 10 minutes. Filter through cotton and wash with 20 ml. of 50% ethanol. Add 1 ml. of 1:1 hydrochloric acid and let the solution come to room temperature. Add 1 ml. of 0.25% sodium nitrite solution. After 10 minutes in the dark, add 1 ml. of 2.5% ammonium sulfamate solution. Let it stand 10 minutes in the dark. Add 2 ml. of 1% solution of *N*-1-naphthalenediamine dihydrochloride. After 20 minutes in the dark, add 4 ml. of chloroform. Shake, and filter the chloroform through cotton. Read at 565 m μ against chloroform.

PARATHION, *O,O*-DIETHYL-*O-p*-NITROPHENYL THIOPHOSPHATE

The purple-red color formed by the reaction between parathion and (2-diethylaminoethyl)-1-naphthylamine oxalate is used to determine parathion in the range of 0.5-5 mg. per ml. The sample is extracted with benzene reduced and diazotized prior to color development.⁸⁰ A similar color is developed with *N*-1-naphthylethylenediamine hydrochloride.⁸¹ The method is also applicable to parathion-methyl.

Both parathion and *p*-nitrophenol are separated from the sample by extraction into benzene. Interferences are removed by passage through alumina. The parathion and *p*-nitrophenol are purified separately on paper chromatograms. *p*-Nitrophenol is read separately at 390 m μ . All steps of the determination should be carried out in subdued light. Beer's law is followed for 0.05-0.4 mg. per 10 ml.

Parathion may be extracted from water, which contains no substances

⁷⁹ Donald A. George, *J. Assoc. Official Agr. Chemists* **46**, 960-3 (1963).

⁸⁰ Hiroyasu Hamada, *Shikoku Acta Med.* **7**, 389-94 (1955); *ibid.* **8**, 1-6 (1956).

⁸¹ Kurt Erbe, *Acta Pharmacol. Toxicol.* **14**, 173-87 (1958); J. Zachrosnska, *Rocz. Zakl. Hig. Warsaw*, **13**, 277-86 (1962).

soluble in benzene, by benzene, and read directly in the ultraviolet at $280\text{ m}\mu^{82}$ (cf. Vol. IV, p. 25). Another method for parathion involves the acidic hydrolysis of the parathion to free *p*-nitrophenol, which is reduced with zinc and hydrochloric acid to form *p*-aminophenol, which is then photometrically determined.⁸³

A mixture of parathion, methylparathion, *O*-ethyl-*O*-*p*-nitrophenyl phenylphosphonothioate (EPN) and their corresponding phosphate esters (oxons) is separable by paper chromatography. These are extracted from homogenized plant material with methyl cyanide. After being taken up on polyethylene alumina to clean up the solution, they are eluted with 40% aqueous methyl cyanide. Then they are partitioned between water and chloroform and sorbed on magnesol. The eluate with chloroform is hydrolyzed to read the *p*-nitrophenol at $400\text{ m}\mu$.⁸⁴

Procedure—Plant tissue as nitrophenol. Homogenize a 50-gram sample with 100 ml. of acetonitrile for 3 minutes. Centrifuge, and filter through a plug of glass wool. Homogenize the residue with another 100 ml. of acetonitrile for 2 minutes. Centrifuge and decant as before. Evaporate the acetonitrile in a flash evaporator. When frothing ceases, measure the volume of aqueous solution remaining and add acetonitrile to make that reagent 40%. Add 1 ml. of 1:10 hydrochloric acid and mix.

Pass through a column of 15 grams of polyethylene-coated alumina. Collect the effluent at 5-10 ml. per minute. Wash the flask with 20 ml. and 20 ml. of a solution containing 1 ml. of 1:10 hydrochloric acid per 100 ml. of 40% acetonitrile. Add each of these to the column when the liquid closely approaches the top of the alumina. Then add the other 60 ml. of the 40% acetonitrile, and collect the effluent until the column is dry.

Evaporate the acetonitrile from the effluent in a flash evaporator at 35° and transfer to a separatory funnel. Wash the flask with four 50-ml. portions of chloroform and use each for extraction of the main solution.

Prepare a column of 5 grams of magnesol and 30 grams of anhydrous sodium sulfate. Pass the chloroform extract through this into a vacuum collection flask. Wash the flask and column with 25, 25, and 25 ml. of chloroform. Evaporate the chloroform in a flash evaporator at 35° . Take up in chloroform and dilute to 10 ml.

⁸² Hirohisa Kita, Hiroshi Maeda, Bunichi Hanazaki, Masakazu Kawai and Toshihiko Takazawa, *Bull. Tokyo Med. Dental Univ.* **4**, 379-83 (1957).

⁸³ P. Abrashieva and L. Stoicheva, *Nauch. Trudove Vissheya Med. Inst. Sofia* **4**, No. 3, 45-54 (1956); J. Kubistova, *Pracovní Lekar* **15**, 108-10 (1963).

⁸⁴ D. E. Coffin and W. P. McKinley, *J. Assoc. Official Agr. Chemists* **46**, 223-8 (1963); K. Helrich, *ibid.* **47**, 242-4 (1964).

Evaporate 1-2 ml. of the purified extract in a stream of nitrogen. To the residue, add 0.2 ml. of 30% hydrogen peroxide. Heat at 35° for 15 minutes. Add 4.8 ml. of 0.28% potassium hydroxide solution and continue to heat at 35° for 20 minutes. Centrifuge, and read the yellow layer at 400 m μ . The standard for comparison is *p*-nitrophenol, to which the test substances have been converted. The result includes parathion, methylparathion, and similar compounds.

By N-(1-naphthyl)ethylenediamine. Biological materials. Homogenize the sample and acidify a 10-gram portion with 1 ml. of 1:2 sulfuric acid. Triturate with 20 grams of Hyflo Supercel to a homogeneous mixture and extract with benzene in a Soxhlet extractor for 4 hours. Concentrate the extract in a current of air to 25 ml. Filter if necessary.

Prepare a 15 \times 100-mm. column of acid alumina and cover with 50 mm. of anhydrous sodium sulfate. Wash the column with 25 ml. of benzene. When the level of the liquid just reaches the surface of the column, add the sample and adjust the flow rate to not more than 3 ml. per minute. Rinse the flask containing the sample with two 5-ml. portions of benzene and add the rinsings to the column. Wash the sample through with 20 ml. of benzene, added in small portions. Do not allow the level of the liquid to fall below the top of the column. Discard the benzene eluates. Elute with 50 ml. of ether and collect the eluate containing parathion. Elute with 50 ml. of methanol and collect the eluate separately containing *p*-nitrophenol.

Parathion. The concentration tube is a short, wide tube with a narrow graduated lower part and a detachable air inlet tube. Using the concentration tube, concentrate the parathion in ether in a current of air to a small volume. Adjust to 0.4 ml. with chloroform and use this solution for paper chromatography.

Impregnate two strips of Whatman No. 1 paper, 12 cm. wide, by immersing in a 25% dimethylsulfoxide solution in toluene. Blot between sheets of filter paper, dry at 70° for 2 minutes, and repeat the immersing, blotting, and drying once more. Promptly apply to each strip two 0.1-ml. aliquots of the sample, corresponding to 2.5 grams of the sample, spreading the solution in the form of bands 25 mm. in length and 20 mm. apart. To serve as a control or as guide strip, apply 0.02 ml. of parathion standard containing 1 mg. of technical parathion per ml. of benzene along the bands of each paper. Immediately develop the chromatograms until the solvent front has reached 15-20 cm. from the origin, using iso-octane

saturated with dimethylsulfoxide as the mobile solvent. Dry in air at room temperature and use one paper for the qualitative test and the other for the determination.

Spray one paper with 0.3% potassium hydroxide solution in ethanol and heat at 100° in an oven for 5 minutes to hydrolyze the parathion to *p*-nitrophenol. Parathion is indicated by the appearance of a canary yellow band on the sample chromatogram corresponding in shade and location to an R_F value of 0.7.

If the spot test is positive, treat the excised guide strip of the second paper with 0.3% potassium hydroxide solution in ethanol. Guided by the appearance of the *p*-nitrophenol spot, localize and cut out the parathion area of the untreated part of the sample chromatogram. To serve as a blank, also cut out from the same chromatogram a similar section containing no applied material. Suspend the paper in a glass hook attached to the lower coil of a reflux condenser fitted to a small flask. Add 3 ml. of ethanol through the condenser and boil for 20 minutes, thus extracting the paper by the condensing ethanol vapors.

Through the condenser, add 4 drops of 1:1 hydrochloric acid, 3 ml. of water, and a granule of zinc. Reflux for 10 minutes. Quantitatively transfer the solution of reduced parathion without the zinc granule to a 10-ml. volumetric flask with the aid of 1 ml. of water. Add 4 drops of 0.25% sodium nitrite solution and mix. After 10 minutes, add 4 drops of 2.5% ammonium sulfamate solution and mix. After 10 minutes, add 4 drops of 1% *N*-(1-naphthyl)ethylenediamine dihydrochloride solution and mix. Dilute to 10 ml. with water. Read the magenta-colored solution after 10 minutes at 555 $m\mu$ against the blank solution prepared from the cut-out paper.

p-Nitrophenol. Evaporate the methanol eluate to 10 ml. Add 1 ml. of 20% sodium hydroxide solution and 50 ml. of water. Remove lipids by extracting with two 10-ml. portions of ether, and discard the ether extracts. Acidify the aqueous phase with 1:2.5 sulfuric acid and extract with one 20-ml. portion and two 10-ml. portions of ether. Wash the combined ether extracts with 10 ml. of 25% sodium chloride solution and dry with anhydrous sodium sulfate. Filter, and wash the sodium sulfate with ether. Using the concentration tube described in the parathion section, evaporate the sample to near dryness in a current of air and adjust the volume to 0.4 ml. with methanol. Add a few drops of chloroform, if necessary, to clear the solution.

Prepare the paper chromatography as described in the parathion sec-

tion, starting at "Impregnate two strips . . ." and ending with "in the form of bands 25 mm. in length and 20 mm. apart." To serve as a control, apply 0.02 ml. of *p*-nitrophenol standard, containing 1 mg. of *p*-nitrophenol per ml. in 95% ethanol, along the bands of each paper. Let the paper stand for 1-2 hours to allow for equilibration. Develop the chromatogram with *n*-butanol saturated with 1:2 ammonium hydroxide until the solvent front has advanced about 20 cm. from the starting line. Air dry the paper for 30 minutes. *p*-Nitrophenol is indicated by the appearance of a yellow band on the sample chromatogram, identical in appearance and location to an R_F value of 0.5, with the guide spot. Cut out the yellow band of the sample chromatogram and elute the nitrophenol by refluxing with methanol. Follow the procedure under parathion, starting at "Suspend the paper on a glass hook . . .," using methanol in place of ethanol, and ending with "thus extracting the paper by the condensing ethanol vapors." Add 1 drop of 20% sodium hydroxide solution to the methanol, dilute to 10 ml. with methanol, and read at 390 $m\mu$.

Tissue by a naphthylamine oxalate. Extract the sample with 10 volumes of benzene. Concentrate the extract at 100° to one-tenth its original volume and evaporate to dryness at room temperature. Dissolve the residue in 2 ml. of 95% ethanol. Add 1 ml. of 1:10 hydrochloric acid and 0.3 gram of zinc dust, and reduce by heating at 40° for 40 minutes. Cool and filter. Add 5 drops of 1:9 hydrochloric acid and 5 drops of 0.2% sodium nitrite solution. After 5 minutes, add 5 drops of 25% urea solution. Let stand at room temperature for 15 minutes. Add 3 drops of 0.2% (2-diethylaminoethyl)-1-naphthylamine oxalate solution, and read after 40 minutes at 560 $m\mu$.

1-(2-NITRO-4-METHYLPHENYLAZO)-2-NAPHTHOL, D & C RED No. 35

This is determined simultaneously with 1-(4-methylphenylazo)-2-naphthol. See Chapter 6, page 456.

NITRO AND NITROSO DIPHENYLAMINES

Nitro and nitroso diphenylamines are quite soluble in *N,N*-dimethylformamide (DMF). They are, in some cases, read directly; in others, they are developed with tetraethyl ammonium hydroxide, with or without

addition of fluorene.⁸⁵ Many have been read in absolute ethanol.⁸⁶ *p*-Nitrosodiphenylamine is also determined by palladous chloride, using the reverse of a well-developed reaction.⁸⁷

The absorption peak of fluorene at 555 $m\mu$ interferes somewhat at low concentrations of the compounds. The color, except with the palladous chloride reagent, develops instantaneously. Only in the case of hexanitrodiphenylamine does the addition of fluorene improve the color stability. The colors with mono and dinitro compounds are stable for at least one hour. The color for 2,4-dinitrodiphenylamine is stable for at least 24 hours. The color developed with hexanitrodiphenylamine discolors rapidly unless fluorene is added; then it shows fair stability for at least an hour. Picric acid must be read immediately with either reagent, as discoloration occurs rapidly. It is included, although not a diphenylamine derivative, because it can be considered as the end product of a series of nitrosations and nitrations occurring in storage of smokeless powder or other explosives containing the nitro group.

Beer's law holds up to 0.6 mg. per 50 ml. for 2-nitrodiphenylamine, up to 0.4 mg. for the dinitro compounds and picric acid, and up to 0.2 mg. for the hexanitro and 4-nitro compounds. *N*-Nitrosodiphenylamine does not react with either reagent, nor does it absorb in the visible range. It has an absorption peak at 284 $m\mu$, which can be read up to 0.6 mg. per 50 ml. of DMF. *p*-Nitrosodiphenylamine gives less absorption with the reagents than without them. It is therefore read directly up to 0.3 mg. per 50 ml. of DMF. Alternatively, it is developed with palladous chloride. In that case, the presence of hydrochloric acid is essential to obtaining the coordination complex.

The molar absorbances of various derivatives are shown in Table 3. In many cases, the molar absorbance is substantially greater in DMF than in ethanol. Beer's law is followed by 10 mg. or less of 2,2',4,4',6,6' hexanitrophenylamine in 10 ml. of dioxane, carbon tetrachloride, benzene, and ethyl ether.⁸⁸ The color is orange or red. Organic amines interfere.

⁸⁵ Walter Selig, *U. S. Atomic Energy Comm. UCRL-6903*, 13 pp. (1962); cf. E. Sauricki and T. W. Stanley, *Analyt. Chim. Acta* **23**, 551-6 (1960).

⁸⁶ W. A. Schroeder, E. W. Malmberg, L. L. Fong, K. N. Trueblood, J. D. Landerl, and E. Hoerger, *Ind. Eng. Chem.* **41**, 2818-27 (1949); W. A. Schroeder, P. E. Wilcox, K. N. Trueblood and A. O. Dekkar, *Anal. Chem.* **23**, 1740-7 (1951).

⁸⁷ John H. Yoe and L. G. Overholser, *J. Am. Chem. Soc.* **61**, 2058-63 (1939); L. G. Overholser and John H. Yoe, *Virginia J. Sci.* **1**, 162-7 (1940); *Anal. Chem.* **13**, 3227-9 (1941).

⁸⁸ S. Kertes, *Anal. Chim. Acta* **15**, 73-6 (1956).

TABLE 3. MOLAR ABSORBANCES OF SOME DERIVATIVES OF DIPHENYLAMINE IN DIMETHYLFORMAMIDE AND IN ETHANOL

	<i>Dimethylformamide</i>		<i>Ethanol</i> ^{1,2}	
	λ_{\max} (m μ)	$\epsilon \times 10^{-4}$	λ_{\max} (m μ)	$\epsilon \times 10^{-4}$
2-Nitrodiphenylamine	430	0.66	422	0.66
4-Nitrodiphenylamine	396	2.33	390	2.12
2,4-Dinitrodiphenylamine	364-66	1.66	349-51	1.70
2,4'-Dinitrodiphenylamine	408-12	1.42	405	1.39
2,2',4,4',6,6'-Hexanitrodiphenylamine	420-25	3.08	376-79 ^a	1.70
			410-12 ^b	2.90
Picric acid	375-77	1.86	335 ^a	0.54
			357-59 ^b	1.40
<i>p</i> -Nitrosodiphenylamine	425	3.01	405-07 ^a	2.04
			390-92 ^b	2.64
			421	2.75
<i>N</i> -Nitrosodiphenylamine	283-85	0.59	295-96	0.57

^a Solution was acidified with one drop of conc. HCl per 100 ml.

^b Solution was made alkaline with one drop of 12N NaOH per 100 ml.

The reaction of the hexanitro compound with quaternary ammonium compounds has been studied.⁸⁹ The color body is extracted with chloroform to read at 420 m μ . Amines do not interfere.

Procedure—*Direct reading.* Dissolve an appropriate weight of sample in DMF and read at the wave length of maximum absorption as shown in Table 4.

By tetraethylammonium hydroxide. To an appropriate solution of the compound in DMF, add 1 ml. of 10% aqueous tetraethylammonium hydroxide. Dilute to 50 ml. with DMF and read at once against DMF.

With added fluorene. Add 5 ml. of 1% solution of fluorene to the sample. Then add 1 ml. of 10% aqueous tetraethylammonium hydroxide and dilute to 50 ml. with DMF. Read at once against DMF. This variation is recommended only for 2,2',4,4',6,6'-hexanitrodiphenylamine.

p-Nitrosodiphenylamine. As a reagent, dissolve 0.174 gram of palladous chloride in 2 ml. of concentrated hydrochloric acid. Dilute to 100 ml. with DMF. This reagent is stable for 4 days. Add 5 ml. of this reagent to the sample in DMF and dilute to 50 ml. with DMF. After 30

⁸⁹ G. Schill and B. Danielsson, *ibid.* 21, 248-54 (1956); G. Schill, *ibid.* 21, 341-52 (1959).

TABLE 4. ABSORPTION MAXIMA AND MOLAR ABSORBANCES FOR SOME NITRO DERIVATIVES OF DIPHENYLAMINE IN DIMETHYLFORMAMIDE

	Color with reagents	Tetraethylammonium hydroxide only		Fluorene and Et ₄ NOH	
		λ_{\max} (m μ)	$\epsilon \times 10^{-4}$	λ_{\max} (m μ)	$\epsilon \times 10^{-4}$
2-Nitrodiphenylamine	Purple	540	0.88	550	0.91
4-Nitrodiphenylamine	Rose	500	3.27	500	4.09
2,4-Dinitrodiphenylamine	Amber	435	1.97	435	1.97
2,4'-Dinitrodiphenylamine	Purple	510	2.42	510	2.42
2,2',4,4',6,6'-Hexanitro- diphenylamine	Amber	508	6.92	508	6.71
Picric acid	Amber	492	2.39	492	2.25
<i>p</i> -Nitrosodiphenylamine	Amber	445	2.95	435	3.08
<i>N</i> -Nitrosodiphenylamine	Clear	No reaction		No reaction	

minutes, read at 500 m μ against a reagent blank. The color is stable for at least an hour.

DINITROBENZENES

o-Dinitrobenzene on reduction gives a violet product extractable with butanol. *p*-Dinitrobenzene gives a yellow product not so extracted. Reduction may be by stannous chloride in alkaline solution, but the use of ascorbic acid or glucose is more convenient.⁹⁰

Procedure—To 5 ml. of sample solution containing up to 0.1 mg. of *o*-dinitrobenzene and up to 0.02 mg. of *p*-dinitrobenzene, add 5 ml. of 8% sodium hydroxide solution. Mix with 5 ml. of 0.88% ascorbic acid solution and let stand for 5 minutes. Read the ortho isomer at 550 m μ and the para isomer at 400 m μ . The meta isomer is unaffected.

An alternative reducing agent is 5 ml. of 1% glucose solution, in which case, the reading is taken after 30 minutes. In either case, the reduction product of the ortho isomer may be extracted with butanol if desired.

DINITRO COMPOUNDS

A method developed for 3,5-dinitro-*o*-toluamide is applicable to many other dinitro compounds. The reagents are dimethylformamide and methylamine.⁹¹ Some mononitro compounds also react. Data in Table 5 show the color of the complex and its absorbance at 550 m μ .

⁹⁰ Y. Azumori, Morita, *J. Chem. Soc. Japan, Pure Chem. Sect.* 84, 806-16 (1963).

⁹¹ Grant N. Smith, *Anal. Chem.* 32, 32-37 (1960).

TABLE 5. SPECIFICITY OF DIMETHYLFORMAMIDE-METHYLAMINE REACTION

<i>Compound</i>	<i>Color of complex</i>	<i>Ab-sorb-ance at 550 mμ</i>	<i>Compound</i>	<i>Color of complex</i>	<i>Ab-sorb-ance at 550 mμ</i>
3,5-Dinitrobenzamide	Purple	0.930	N-Ethyl-3,5-dinitro- <i>o</i> -toluamide	Purple	0.510
2,6-Dinitrobenzamide	Purple	0.035	<i>N,N</i> -Dimethyl-3,5-dinitro- <i>o</i> -toluamide	Green	0.146
3,4-Dinitrobenzamide	Yellow	...	<i>N,N</i> -Diethyl-3,5-dinitro- <i>o</i> -toluamide	Green	0.135
2,5-Dinitrobenzamide	Yellow	...	3,5-Dinitro- <i>o</i> -toluic acid	Colorless	...
3,5-Dinitro- <i>o</i> -toluamide	Purple	0.680	5-Nitro- <i>o</i> -toluamide	Colorless	...
4,6-Dinitro- <i>o</i> -toluamide	Purple	0.660	3-Nitro- <i>o</i> -toluamide	Colorless	...
4,5-Dinitro- <i>o</i> -toluamide	Orange	0.002	3-Nitro- <i>o</i> -toluic acid	Colorless	...
3,5-Dinitro- <i>p</i> -toluamide	Green	0.017	Ammonium 3,5-dinitro- <i>o</i> -toluate	Colorless	...
2,6-Dinitro- <i>p</i> -toluamide	Purple	0.080	3,5-Dinitrophthalic acid	Blue	...
4,6-Dinitro- <i>m</i> -toluamide	Blue	0.190	2,4-Dinitrotoluene	Blue	...
2-Ethyl-3,5-dinitrobenzamide	Purple	0.790	2-Methyl-5-nitroaniline	Yellow	...
2-Isopropyl-3,5-dinitrobenzamide	Purple	0.700	<i>p</i> -Dimethylamino-benzaldehyde	Colorless	...
2-Amino-3,5-dinitrobenzamide	Orange	0.310	2,4-Dinitrophenylhydrazine	Green	...
2-Hydroxy-3,5-dinitrobenzamide	Yellow	0.003	Picric acid	Orange	...
2-Chloro-3,5-dinitrobenzamide	Orange	0.170	<i>p</i> -Nitroacetanilide	Yellow	...
4-Amino-3,5-dinitrobenzamide	Yellow	...	2,4-Dinitrophenylurea	Orange	...
4-Chloro-3,5-dinitrobenzamide	Yellow	...	4,4'-Dinitrocarbanilide	Yellow	...
2-(2,4-Dinitrophenyl)-acetamide	Green	...	2-Ethyl-5,7-dinitro-benzoxazole	Red	...
3,5-Dinitro- <i>o</i> -toluoyl chloride	Colorless	...	2-Methyl-5,7-dinitro-benzoxazole	Red	...
3,5-Dinitro- <i>o</i> -tolunitrile	Colorless	...	2,4-Dinitrophenol	Yellow	...
3,5-Dinitro- <i>o</i> -toluene-sulfonic acid	Green	...	2,4-Dinitroaniline	Red	...
3,5-Dinitro- <i>o</i> -anisamide	Orange	0.195	Diphenylamine	Orange	...
<i>N</i> -Methyl-3,5-dinitrobenzamide	Purple	0.820	Picramic acid	Orange	...
<i>N</i> -Isobutyl-3,5-dinitrobenzamide	Purple	0.720	Nitrobenzene	Pink	...
<i>N</i> -Methyl-3,5-dinitro- <i>o</i> -toluamide	Purple	0.820	Nicarbazin	Yellow	...
			3-Nitro-4-hydroxyphenylarsonic acid	Yellow	...
			Nitrofurazone	Yellow	...

Procedure—Dilute the sample in acetone to about 0.5 mg. of test substance per ml. To 1 ml., add 24 ml. of dimethylformamide at not over 20°. Dilute to 50 ml. with 40% aqueous methylamine solution precooled to 3–5°. Exactly 3 minutes after mixing, read at 390 and 550 $m\mu$, often against a standard.

m-DINITRO COMPOUNDS

The reaction of diethylamine in dimethylsulfoxide is suitable for reading many meta-dinitro compounds, as shown in Table 6. The diethylamine

TABLE 6. COLOR REACTIONS OF VARIOUS META-DINITRO COMPOUNDS

Compound	Color after diethylamine	Beckman DU readings at 45–75 minutes	
		Wave length of max. absorption, $m\mu$	Absorptivity
<i>m</i> -Dinitrobenzene	Purple	575	785
3,5-Dinitrobenzamide	Purple	570	16,065
3,5-Dinitrobenzoic acid, methyl ester	Purple	555	16,960
<i>N</i> -Methyl-3,5-dinitrobenzamide	Purple	566	15,410
3,5-Dinitrobenzhydrazide	Blue	575	14,610
3,5-Dinitrobenzoic acid, <i>n</i> -butyl ester	Purple
3,5-Dinitrobenzoic acid, isopropyl ester	Purple	555	14,610
3,5-Dinitrohippuric acid	Purple	565	14,150
3,5-Dinitrobenzoyl- <i>p</i> -nitroaniline	Amber	555	5,810
<i>N,N</i> -Di-(2-hydroxyethyl)-3,5-dinitrobenzamide	Purple
2,4-Dinitrophenylhydrazine	Red-orange
2,4-Dinitrophenylacetic acid	Green	695	775
2,4-Dinitrotoluene	Green	695	894
2,4-Dinitrophenyl thiocyanate	Yellow-green	430	12,530
2,2',4,4'-Tetranitrophenyl	Pink	640	26,370
Bis-(2,4-dinitrophenyl)disulfide	Orange	650	36,850
3,7-Dinitrophenothiazine sulfoxide	Red
<i>N'</i> -(2,4-Dinitrophenyl)- <i>N</i> ² -phthaloyl	Amber
2,7-Dinitroanthraquinone	Purple	535	1,090

should not be pure; it is artificially aged. Compounds that do not give a visible color with this reagent are: 3,5-dinitrobenzoic acid; 2,6-dinitro-4-chloroaniline; 2,6-dinitrohydroquinone-4-monoacetate; 3,5-dinitrosalicylic acid; 3,5-dinitrosalicylic acid, methyl ester; 3,5-dinitrosalicylamide;

2,4-dinitroaniline; 2,4-dinitrodiethylaniline; 2,4-dinitrobenzene sulfonic acid; 4,6-dinitro-*o*-cresol; 5,7-dinitro-8-quinolinol; 2,4-dinitrophenol; *N,N*-bis-(2-hydroxyethyl)-2,4-dinitroaniline; 2,2',4,4'-tetranitrocarbani-
lide; and 6,8-dinitro-2,4-(1H,3H)-quinazolinedione.

All the 3,5-disubstituted compounds that react with the reagents have a maximum absorption in region of 540 to 575 $m\mu$. The 2,4-disubstituted compounds, with the exception of 2,4-dinitrophenyl thiocyanate ($\lambda_{\max} = 430 m\mu$), absorb at 640 to 695 $m\mu$.

Procedure—As an aging procedure, reflux 40 grams of sodium or potassium fluosilicate with 1 liter of dry diethylamine for 48-72 hours. To 8 ml. of sample containing about 0.1 mg. of the compound in dimethylsulfoxide, add 2 ml. of the treated diethylamine. Read at the wave length indicated in Table 6.

DINITROPHENYLAMINO GROUPS

Fluorodinitrobenzene reacts with amino groups, whether of amino acids or proteins, to form a 2,4-dinitrophenylamino compound. The reaction should have broader applications. The 2,4-dinitrophenylamino compound forms an intense red color when sodium borohydride is added to the aqueous solution. This reaction permits the measurement of the number of dinitrophenylamino groups present in papain, lysozyme, ribonuclease, and tobacco mosaic virus protein.⁹² The reaction is highly specific, with a precision of 2%, and can estimate 0.01-0.06 micromole of dinitrophenyl compound per milliliter.

As interference, 2,4-dinitroaniline reacts under the conditions of the procedure, while *o*-, *m*-, and *p*-nitroanilines, 2,4-dinitrophenyl aryl or alkyl ethers, and 2,4-dinitrophenyl imidazole and pyrrolidine derivatives do not. The color of the dinitrophenyl derivative is also read at 360 $m\mu$ and 300 $m\mu$ in sodium bicarbonate solution.⁹³

Procedure—Add 1 micromole of reactive amino compound to 2 ml. of 2.5% sodium bicarbonate solution. Add 4 ml. of ethanol containing 50 mg. of fluorodinitrobenzene. Let stand at room temperature for a measured interval, shaking occasionally. Add an equal volume of acetone and centrifuge the precipitate. Wash the precipitate with three 3-ml.

⁹² L. K. Ramachandran, *Anal. Chem.* **33**, 1074-8 (1961).

⁹³ I. A. Vaintraub, Tr. po Khim. Prirodn. Soedin., Kishinevsk. Gos. Univ. **4**, 89-91 (1961).

portions of acetone containing 2% of concentrated hydrochloric acid. Wash with two 5-ml. portions of water containing 2% of concentrated hydrochloric acid. Complete the washing with 5 ml. of acetone. Combine the supernatant liquids and recover any flocculent precipitate present. Wash any recovered precipitate. Dilute the dinitrophenylamino compound with 60% aqueous urea and let stand at 40° for 1 day. Warm to dissolve if necessary.

To 1 ml. of the solution containing about 0.5 micromole of dinitrophenyl compound add 5 ml. of water, 1 ml. of 2% sodium bicarbonate solution, and 1 ml. of 0.5% sodium borohydride solution. Prepare a blank by following the above procedure, replacing the sample with 1 ml. of 1% sodium borohydride. Read within 35-60 minutes at 420 m μ .

3,5-DINITRO-*o*-CRESOL

A specific reaction of cyanide with dinitrophenol groups has been applied to dinitro-*o*-cresol.⁹⁴ The primary application is to residues on food crops.

Procedure—Macerate the sample. Extract 100-gram portions twice with 150-350 ml. portions of 0.04% sodium hydroxide solution. Filter the combined extracts and acidify with 1:10 hydrochloric acid. Add a few drops of isoamyl alcohol. Extract with 150 ml. and 100 ml. of petroleum ether. Combine these extracts and evaporate the solvent at 100°. Take up the residue in 5 ml. of ethanol. Add 0.5 ml. of propanol and two drops of 10% aqueous potassium cyanide. Heat at 100° for 10 minutes, cool, and dilute to a known volume with ethanol. Read at 350 m μ .

DINOSEB, 2-SEC-BUTYL-4,6-DINITROPHENOL

Dinoseb is read in ethyl methyl ketone as the sodium salt.⁹⁵

Procedure—*Potatoes*. Homogenize a 100-gram sample with 100 ml. of chloroform containing 5 grams of chloroacetic acid. Filter. Wash the residue with 20, 20, and 20 ml. of chloroform. Pass the combined chloroform extracts through anhydrous sodium sulfate. Evaporate the dried chloroform extract to dryness. Take up the residue in 5 ml. of chloroform.

⁹⁴ E. Heimisch and G. Panser, *Nachrbl. Deut. Pflanzenschutzdienst* (Berlin) 17, (4/5), 85-91 (1963).

⁹⁵ J. A. Potter, *Analyst* 88, 651-3 (1963).

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664493
7/3/65

Shake this vigorously with 10 ml. of 2% sodium carbonate solution. Discard the chloroform layer.

To 5 ml. of the solution, add 1 gram of sodium chloride, 2 ml. of 2% sodium carbonate solution, and 5 ml. of ethyl methyl ketone. Shake vigorously and separate the ketone layer. Read at 379 m μ . Make a portion of the extract acid with hydrochloric acid and read at 379 m μ . Subtract this correction for nonphenolic plant pigments from the first reading.

2,4-DINITROPHENYL THIOCYANATE

The violet color developed by 2,4-dinitrophenyl thiocyanate with ammonia is suitable for photometric reading.⁹⁶

Procedure. Dilute a sample containing 0.04-0.1 mg. of 2,4-dinitrophenyl thiocyanate in acetone to about 8 ml. with the same solvent. Add 0.8 ml. of concentrated ammonium hydroxide and dilute to 10 ml. with acetone. Let stand 30 minutes for color development and read at once at 510 m μ .

3,5-DINITROBENZAMIDE

A readable color is formed when dinitrobenzenes are dissolved in acetone. Likewise, reaction of 3,5-dinitrobenzamide in acetone with concentrated ammonium hydroxide or liquid ammonia produces a blue color.⁹⁷ The procedure determines 0.1-0.5 mg. of amide in feed samples. 3-Nitro-4-hydroxyphenylarsonic acid, *N,N'*-di-(3-nitrobenzene-sulfonyl)ethylene-diamine, *N'*-acetyl-*N*-(4-nitrophenyl)sulfanilamide and nitrophenide do not interfere.

Sample—*Feeds containing 0.025% of 3,5-dinitrobenzamide.* Mix a 2-gram sample with 100 ml. of absolute methanol and shake well. Shake intermittently for 30 minutes and filter. Evaporate a 20-ml. portion of the filtrate to dryness at 100°. Add 10 ml. of acetone and warm to dissolve. Carefully transfer to a 25-ml. volumetric flask. Add 5 ml. of concentrated ammonium hydroxide to the beaker and swirl to dissolve any remaining residue. Rinse the beaker with an additional 10 ml. of acetone, add the acetone to the volumetric flask, and dilute to 25 ml. with acetone. Let the solution stand under a towel for 45 minutes and read at 580 m μ .

⁹⁶ Viktor Andriská and Gyorgyi Bruckner, *Magyar Kem. Folyoirat* 67, 301-3 (1961).

⁹⁷ Herman Beckman and Jeanne DeMottier, *J. Agr. Food Chem.* 7, 280-2 (1959)

Feeds. Samples containing 0.15% of 3,5-dinitrobenzamide. Mix a 2-gram sample with 100 ml. of absolute methanol and shake intermittently for 30 minutes. Filter, and dilute a 10-ml. portion of the filtrate to 50 ml. with absolute methanol. Treat as above, starting with "Evaporate a 20-ml. portion of the filtrate . . ."

ZOALENE, 3,5-DINITRO-*o*-TOLUAMIDE

3,5-Dinitro-*o*-toluamide reacts with dimethylformamide in the presence of methylamine to form a purple complex with a primary absorption peak at 550 $m\mu$ and a secondary peak at 390 $m\mu$.⁹⁸

The absorbance of an unknown sample of 3,5-dinitro-*o*-toluamide in dimethylformamide-methylamine is compared with the absorbance of a reference sample in the same solvent at 550 $m\mu$. The absorbance is also compared at 390 $m\mu$. By comparing the readings, it is possible to determine the purity of the unknown sample in terms of the reference. The isomers of 3,5-dinitro-*o*-toluamide, which are 4,6-dinitro-*o*-toluamide and 4,6-dinitro-*m*-toluamide, both form the characteristic purple complex with the reagents. The isomers may be distinguished by their secondary absorption peaks of 350 $m\mu$ and 400 $m\mu$ respectively, and may be determined by solving simultaneous binomial equations. In the case of three isomers, trinomial simultaneous equations are set up based on the readings at the respective primary and secondary absorption peaks.

Both 2,4-dinitrobenzamide and 3,5-dinitrobenzamide form purple complexes under the conditions of the procedure. The introduction of an alkyl group in the 2-position of 3,5-dinitrobenzamide shifts the absorption peak at the 350 $m\mu$ region and decreases the color intensity. The secondary peak at 390 $m\mu$ is not affected.

Introduction of an activating group in the 2-position of dinitrobenzamide shifts the primary peak and decreases color intensity. The secondary peak is also shifted. *N*-Monoalkyl substitution of 3,5-dinitro-*o*-toluamide results in compounds that form purple complexes with the reagents, while *N*-dialkyl substitution results in compounds that form green complexes. The purple complexes have absorption peaks at 390 and 550 $m\mu$, while the green complexes are read at 410 to 415 $m\mu$, thus making it possible to distinguish *N*-monosubstituted dinitro-*o*-toluamides from *N*-dialkyl-substituted dinitro-*o*-toluamide compounds.

Only compounds that have two nitro groups in the meta position and

⁹⁸ Grant N. Smith, *Anal. Chem.* 32, 32-7 (1960); *J. Agr. Food Chem.* 8, 224-6 (1960).

that possess a carboxamide group form the purple complex under the condition of the procedure. A methyl group inhibits the reaction only when it is between the nitro groups. Small concentrations of formic, hydrochloric, and sulfuric acids inhibit the reaction. Large quantities of acetone and alcohol also interfere.

High temperatures decrease the color intensity; therefore, the reagent and solvent are cooled before addition. The aqueous methylamine solution should be kept at 3-5° and should be stored in small bottles so that a large space is not present above the solution. The purple color is formed immediately and fades gradually, making it necessary to read the color at a fixed time after formation. Reproducibility depends on accurately following the procedure. Since dimethylformamide solutions of 3,5-dinitro-*o*-toluamide are unstable, a fresh solution of the reference standard should be prepared for each determination.

This coccidiostat is extracted from tissue with acetone and benzene. After chromatographing on an alumina column, it is determined photometrically by 1,3-diaminopropane in the presence of dimethylformamide.⁹⁹ The method was developed primarily for chicken tissue. The original compound and a metabolite, 3-amino-5-nitro-*o*-toluamide, occur. Fat and other extraneous matter pass through the column, while Zoalene in chloroform is retained. The test substance is later eluted with 80% ethanol. The color complex is then formed in absolute ethanol containing 4% of dimethylformamide.

Recoveries are in the 80-90% range. A study using radioactive-tagged Zoalene showed about 95% extraction, about 4% loss on the column. A yellow color precedes the Zoalene on the column and serves as an indicator, but Zoalene is coming through when the yellow starts toward its maximum. The absorption due to the yellow tissue pigments is insignificant at 560 m μ .

The tissue must not stand at room temperature, as a very significant enzymatic destruction of the compound occurs. This is avoided by immediate freezing of tissue samples with dry ice. No Zoalene is left in either tissue or liver 12 hours after medication of the feed ceases.

Procedure—General. Dilute a sample containing 3,5-dinitro-*o*-toluamide in acetone, so that the final concentration is of the order of 0.5 mg. per ml. Prepare a similar reference standard. To 1 ml. of each solution, add 24 ml. of dimethylformamide at 20° and 25 ml. of 40% methylamine solution, which has been cooled to 3-5°. Mix rapidly. Read triplicate

⁹⁹ G. N. Smith, B. J. Thiels, and M. G. Swank, *ibid.* **9**, 197-201 (1961).

samples of the unknown and reference standard at exactly 3 minutes after mixing at both 390 $m\mu$ and 550 $m\mu$. If the unknown sample is pure 3,5-dinitro-*o*-toluamide, the ratio of absorbance of unknown at 390 $m\mu$ to the absorbance of the reference at 390 $m\mu$ will be the same for the sample and the standard.

Feed concentrates. Suspend a 1-gram sample containing about 250 mg. of 3,5-dinitro-*o*-toluamide in 200 ml. of dimethylformamide. Warm slightly and stir continuously for 5 minutes to ensure complete extraction. Cool to 20° and dilute to 1 liter with dimethylformamide. Mix, and allow insoluble feed particles to settle. Dilute a 10-ml. aliquot to 100 ml. with dimethylformamide.

Further dilute a 10-ml. aliquot to 25 ml. with 40% methylamine solution, which has been cooled to and maintained at 3°. Mix thoroughly and read at 550 $m\mu$ exactly 3 minutes after the addition of methylamine. The final solution contains about 0.01 mg. per ml.

Chicken tissue. Grind the frozen sample and put 50 grams in a Mason jar. Add 250 ml. of acetone and blend for 5 minutes. Filter through a bed of Hyflo Supercel on paper in a Büchner funnel. Wash the residue on the funnel with 100 ml. of benzene.

Transfer the paper and residue back to the Mason jar and blend with 250 ml. of benzene for 3 minutes. Filter as before and wash the residue with 100 ml. of benzene. Transfer the total filtrate to a 1000 ml. separatory funnel, using 50 ml. of benzene to wash out the filter flask. Shake well, swirl, and let stand for 30 minutes. Swirl and let stand until 2 layers are well separated, often several hours.

Withdraw the lower aqueous layer and any interfacial debris into a centrifuge bottle. Transfer the organic layer to a beaker. Rinse the separatory funnel with 100 ml. of 1:2 acetone-benzene mixture, adding the washings to the centrifuge bottle. Shake the bottle and centrifuge. Siphon off the aqueous layer and add the organic layer to the beaker containing the prior extract. Evaporate the solvents to 10 ml. under an infrared lamp, using an air jet. Add 100 ml. of chloroform and evaporate to 50 ml. If not clear, due to residual moisture, repeat additions of chloroform and evaporation until it is.

Prepare a chromatographic column from tubing 18 mm. in outside diameter, and 60 cm. long, having a teflon stopcock and a 250-ml. glass-stoppered bulb at the top. The inside alumina column should be of 80-200 mesh Alcoa grade F-20. Insert a plug of glass wool in the bottom of the

column. Add 60 grams of alumina, gently shaking or tapping to pack as uniformly as possible. With the stopcock open, wash the column with 100 ml. of chloroform, stopping, as usual, at the solvent level just above the alumina. Use 50, 50, and 50 ml. of chloroform to wash the sample into the column and to wash the column. Discard all the effluents.

Elute the Zoalene with 90 ml. of 80% ethanol. Discard the first 30 ml. of effluent and collect the next 50-60 ml. containing the Zoalene. Evaporate under a heat lamp with an air jet until it no longer flows. Do not heat too long or to absolute dryness.

Add 5 ml. of 4% dimethylformamide in absolute ethanol. Warm, with stirring, at 45-50°. A trace of insoluble matter may dissolve in the next step. Add 5 ml. of clear, colorless 1,3-diaminopropane to produce the color. If not clear after 5 minutes, filter through a small fluted filter. Exactly 10 minutes after addition of the color reagent, read at 560 m μ . If the color is too intense, dilute with the reagents.

ANOT, 3-AMINO-5-NITRO-*o*-TOLUAMIDE

This metabolite of Zoalene is liberated from chicken tissue by enzymatic digestion. Thereafter, the extract with acetone and chloroform is separated from extraneous materials, such as fats and pigments, by chromatographing on an alumina column. After elution, a further purification is carried out on a Dowex 50W-X8 ion-exchange column. The resulting eluate is diazotized and coupled with *N*-1-naphthylethylenediamine dihydrochloride.¹⁰⁰

Ficin at pH 4 is the most effective enzyme for liberation of the metabolite from the tissue. Heating to 70-80° after digestion promotes extraction. There is no significant conversion of Zoalene to ANOT during the enzymatic digestion. Most of the ANOT disappears from both muscle and liver within 12 hours after medication of feedings is stopped.

Procedure—Grind the frozen tissue and blend 50 grams with 125 ml. of water and 15 ml. of 1:11 hydrochloric acid in a Mason jar for 5 minutes. Add 5 grams of ficin, a proteolytic enzyme, and continue to mix for 2 minutes. Seal the jar and incubate at 30° for 24 hours.

Heat at 70-80° for 30 minutes and cool to room temperature. Neutralize by cautious addition, with stirring, of 10 grams of sodium bicarbonate. Add 500 ml. of acetone and blend for 5 minutes. Filter the decantate on

¹⁰⁰ B. J. Thiels, G. N. Smith, and J. L. Bivert, *J. Agr. Food Chem.* **9**, 201-4 (1961).

a Supercel layer on a Büchner funnel wet with acetone. Blend the residue in the Mason jar with 200 ml. of acetone for 3 minutes, and filter. When filtration is complete, blend the filter pad and paper with 500 ml. of chloroform for 5 minutes. Meanwhile, transfer the acetone extract to a separatory funnel.

Filter the chloroform extract on a Supercel layer on paper in the Büchner funnel. When filtration is complete, wash the filter cake with 250 ml. and 250 ml. of chloroform. Add this filtrate to the acetone extract and add 50 ml. of chloroform-washings of the flask to the combined extracts. Shake the separatory funnel and let stand until 2 layers separate, usually about one hour. Withdraw the chloroform extract and extract the aqueous phase with 200 ml. of chloroform. Add this extract to the prior one.

Evaporate the combined chloroform extracts to about 50 ml. under an infrared lamp with an air jet. If not clear due to residual moisture, add 50 ml. portions of chloroform and evaporate until it is clear.

Follow the technic for Zoalene, from "As a chromatographic column—" through "collect the next 50-60 ml. . . ."

To prepare a Dowex 50W-X8, use the equipment shown in Figure 2. Heat 100 grams of the 200-400 mesh resin at 100° with 800 ml. of 1:1 hydrochloric acid for several hours. Filter on a Büchner funnel and wash until free of acid. Wash with 100 ml. of 80% ethanol and suspend in 250 ml. of 80% ethanol. Pour into the apparatus to give a bed of 4-5 cm. when settled. Wash with 25 ml. of 80% ethanol, stopping as the liquid level approaches the top of the resin. Moderate pressure may be used.

Add the 50-60 ml. of eluate to the prepared column, using moderate air pressure to expedite its passage. Follow the solution with 50 ml. of 80% ethanol and then with 50 ml. of water. The ANOT is now on the resin, so the washings are discarded.

Elute with 45 ml. of 1:2 hydrochloric acid. Add 1 ml. of 0.25 sodium nitrite to the eluate and mix. Let stand for 5 minutes. Add 1 ml. of 1.25% sodium sulfamate solution and mix. After 5 minutes, add 1 ml. of 0.25% *N*-1-naphthylethylenediamine dihydrochloride and dilute to 50 ml. with 1:2 hydrochloric acid. Mix, and after 15 minutes read at 540 $m\mu$ against a reagent blank.

2-NITRO-2-BROMO-1-METHOXY-1-PHENYLPROPANE

2-Nitro-2-bromo-1-methoxy-1-phenylpropane is hydrolyzed in an alcoholic alkaline medium to form a 2-nitro phenylalkane that reacts with

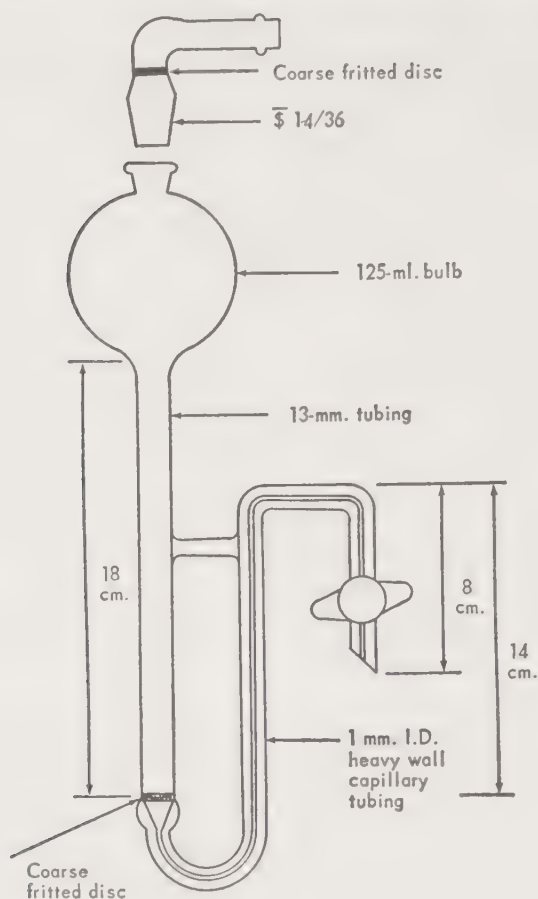


FIG. 2.

Ion exchange column for resin used in determination of 3-amino-5-nitro-*o*-toluamide

acidified ferric chloride to form a colored complex.¹⁰¹ Beer's law is followed up to 2 mg., and as little as 0.025 to 0.05 mg. can be determined.

Sample—Emulsion-type ointments. Dilute a sample in butanol so that a 5-ml. aliquot contains up to 2.5 mg. of 2-nitro-2-bromo-1-methoxy-1-phenylpropane.

Carbowax-base ointments. Extract the sample with butanol. Centrifuge and use a 5-ml. aliquot of the supernatant liquid containing up to 2.5 mg. of 2-nitro-2-bromo-1-methoxy-1-phenylpropane.

¹⁰¹ Lawrence R. Jones and John A. Riddick, *Anal. Chem.* **24**, 569-71 (1952).

Petrolatum-base ointment. Dissolve the sample in 300 ml. of *n*-hexane. Shake thoroughly with 100 ml. of acetonitrile for a few minutes and allow the phases to separate. Drain the lower acetonitrile phase and extract the hexane with three additional 100-ml. portions of acetonitrile. Combine the acetonitrile extracts and evaporate just to dryness. Dissolve the residue in butanol and dilute to 10 ml. with butanol.

Procedure—As a reagent, dissolve 1 gram of ferric chloride hexahydrate, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, in 200 ml. of water, and mix with 100 ml. of 1:19 hydrochloric acid in methanol.

Dilute a butanol solution of the sample with butanol so that a 5-ml. aliquot contains not more than 2.5 mg. of 2-nitro-2-bromo-1-methoxy-1-phenylpropane. Add 1 ml. of 2% sodium hydroxide solution in methanol and heat at 100° for 10 minutes. Cool rapidly and add 3 ml. of ferric chloride reagent. Dilute to 12.5 ml. with anhydrous ethanol and read at 490 $\text{m}\mu$ against a blank prepared from a 5-ml. aliquot of the diluted sample, which has been allowed to stand at room temperature for 10 minutes in the presence of the methanolic sodium hydroxide solution.

**TRIFLURALIN, 2,6-DINITRO-*N,N*-DI-*n*-PROPYL-
 α,α,α -TRIFLUORO-*p*-TOLUIDINE**

The herbicide trifluralin absorbs at 275 and 376 $\text{m}\mu$.¹⁰² Preliminary separation by column chromatography is required. Interferences prevent use of 275 $\text{m}\mu$. None has been found at 376 $\text{m}\mu$.

Procedure—Deactivate Florasil by spreading on blotting paper and exposing to the atmosphere overnight. Successively, in a 25 × 400-mm. chromatographic column with a stopcock, add a glass-wool plug, 25 mm. of anhydrous sodium sulfate, 40 mm. of deactivated Florasil, and 25 mm. of anhydrous sodium sulfate, tapping the column during the additions to compact it. Add 25 ml. of *n*-hexane and release with the stopcock until it comes down to the top of the column.

Dry samples. Place a sample containing about 30 mg. of trifluralin in an extraction thimble under a piece of glass wool. Extract with methanol until no further color comes through and for 30 minutes thereafter. Dilute the extract to 250 ml.

¹⁰² F. J. Holzer, R. E. Scroggs and J. B. Leary, *J. Assoc. Off. Agric. Chem.* 46, 659-62 (1963).

Evaporate a 25-ml. aliquot to dryness at 100°. Take up in *n*-hexane and add to the chromatographic column in several increments. If the residue contains a substantial amount of insoluble matter, add 20 ml. of hexane and 1-2 ml. of water. Swirl until dissolved. Remove the water by adding 3-4 grams of anhydrous sodium sulfate. Evaporate the hexane to about 5 ml. and add to the column.

Wash the column with hexane, discarding the effluent until the trifluralin band has moved approximately three-fourths of the length of the column. Thereafter, collect the eluate and evaporate to about 10 ml. Dilute to 50 ml. with methanol. Read at 376 $m\mu$ against methanol.

Emulsifiable concentrates. Transfer a sample containing about 60 mg. of trifluralin to a separatory funnel, and extract with 25, 25, and 25 ml. of chloroform. Filter through anhydrous sodium sulfate and evaporate just to dryness. Take up in *n*-hexane and dilute to 100 ml. Transfer 5 ml. to the chromatographic column in several increments, and proceed as for dry samples from "Wash the column with hexane . . ."

DINOCAP, 2-(1-METHYLHEPTYL)-4,6-DINITROPHENYL CROTONATE

Dinocap is the active ingredient of Karathane.¹⁰³ It reacts with ethanolic tetraethylammonium hydroxide to give a yellow color absorbing at 425 $m\mu$. The color increases with time until hydrolysis to the phenol is complete.

Dinocap gives a yellow color when dissolved in *N,N*-dimethylformamide.¹⁰⁴ The color develops in 20 minutes and is stable for at least an hour. Beer's law holds up to at least 0.05 mg. in 4 ml. of the reagent. There is no interference by parathion, 2,6-dichloro-4-nitroaniline; 1,3,5-trichloro-2,4-dinitrobenzene; or 1,3-difluoro-4,6-dinitrobenzene. The blank is very low. Recoveries average 88.6% by the technic of cleaning up with a Florasil column, 90% by the alternative sulfuric acid clean-up.

Procedure—*By tetraethylammonium hydroxide. Strawberries.* Individually wash about 50 grams of berries successively in 10 ml., 10 ml., and 10 ml. of benzene. Dry the first 10 ml.-portion of washings with 1-2 grams of anhydrous sodium sulfate. Filter the combined benzene extracts through a 10-mm. layer of anhydrous sodium sulfate on paper. Wash

¹⁰³ E. John Skerrett and E. A. Baker, *Analyst* **87**, 228-9 (1962).

¹⁰⁴ Wendell W. Kilgore and Kin Wa Cheng, *J. Agr. Food Chem.* **11**, 477-9 (1963).

the filter with 5 ml. of benzene. Evaporate the combined washings to dryness. Take up the residue in 8.5 ml. of benzene and add 5 ml. of 95% ethanol. Add 1 ml. of 25% tetraethylammonium hydroxide diluted 1:9 with 95% ethanol. Heat for 30 minutes at 60°, cool, and read at 425 m μ .

Apple leaves. Wash 3 leaves with 10 ml., 10 ml., and 10 ml. of benzene. Evaporate all or an aliquot containing not over 0.2 mg. of dinocap at 200-250 ml. of mercury and not over 60°. Add 1.5 ml. of benzene to dissolve the residue. Add 1.5 ml. of the reagent as described for strawberries. Heat at 60° for 30 minutes and cool. Use 5 ml. of benzene and 5 ml. of 0.4% sodium hydroxide solution to transfer to a separatory funnel. Shake, and separate the aqueous layer. Wash the benzene layer with 5 ml. and 5 ml. of 0.4% sodium hydroxide solution. Acidify the combined aqueous layers with 3 drops of concentrated hydrochloric acid. Extract with 5 ml. of benzene and discard the aqueous layer. Filter the benzene through a 10-mm. layer of anhydrous sodium sulfate. Wash the filter with benzene to give a total volume of filtrate of 8.5 ml. Add 5 ml. of 95% ethanol and 1.5 ml. of the tetraethylammonium hydroxide reagent. Mix, and heat for 30 minutes at 60°. Cool, and read at 425 m μ .

By dimethylformamide. Apples. Weigh 500 grams of well-macerated sample into a 1-gallon tin can, having a baffle for mixing. Add 1 liter of 60-80° hexanes, seal, and roll at 35 rpm for 30 minutes. Allow 10 minutes for dispersion of the emulsion before opening. Decant onto 200 grams of anhydrous sodium sulfate, mix, and filter.

Prepare a 2.5 \times 25-cm. column containing 20 grams at 60-80 mesh Florasil, previously rinsed with 6% ethyl ether in mixed hexanes. Pass a 100-ml. aliquot of the extract through the column. Wash the column with 50 ml. of 6% ether in hexanes. Discard these washings. Then wash successively with 100 and 150 ml. of 6% ether in hexanes, collecting this as the sample.

Evaporate at 40-50° with a rotating flash evaporator. Heating 10 minutes after the solvent disappears and use of a somewhat higher temperature has no significant effect. When the solvent is gone, wash down the sides of the flask with 4 ml. of *N,N*-dimethylformamide. Stopper, and swirl to pick up all of the residue. After 20 minutes, filter through a pledget of glass wool. Read at 444 m μ against the solvent.

As an alternative to the Florasil column, shake 100 ml. of the dried and filtered extract with 20 ml. of concentrated sulfuric acid for 3 minutes. After 5 minutes for the phases to separate, discard the acid layer.

The upper layer will be an emulsion. Wash it with four successive 50-ml. portions of water. Pass the washed sample through a 2.5×25 -cm. column containing about 100 grams of anhydrous sodium sulfate. Rinse the separatory funnel with 50 ml. and 50 ml. of mixed hexanes, and pass these through the column when the level of the sample has fallen to about 1 inch above the sodium sulfate. Wash the column with 50 ml. of mixed hexanes. Continue with the technic in the preceding paragraph, from "Evaporate at 40-50° with . . ."

Grapes or strawberries. Handle as for apples, but do not macerate.

**ACRICID, 2-(1-METHYLPROPYL)-4,6-DINITROPHENYL- β,β -
DIMETHACRYLATE**

This active or closely related ingredient of Acricid and dinocap is washed from leaves with benzene. Thereafter, it is purified by extraction into alkali, and is acidified and extracted by benzene. It is then developed by tetraethylammonium hydroxide.¹⁰⁵

Procedure—Foliage. Wash 3 leaves in three successive 10-ml portions of benzene. Dry the extracts with anhydrous sodium sulfate and filter. Wash the filter with 5 ml. of benzene. Evaporate the filtrate, or an aliquot containing not more than 0.2 mg. of Acricid at 200-250 mm. of mercury and not over 60°, to about 5 ml. Add 1 ml. of ethanol and 1.5 ml. of reagent consisting of a 25% solution of tetraethylammonium hydroxide diluted 1:9 with ethanol. Heat at 60° for 30 minutes and cool. Use 5 ml. of benzene and 5 ml. of 0.4% sodium hydroxide solution to transfer to a separatory funnel. Extract, and separate the aqueous layer. Wash the benzene layer with 5 ml. of 0.4% sodium hydroxide solution. Acidify the aqueous layer and washings with 3 drops of concentrated hydrochloric acid. Extract with 5 ml. of benzene and discard the aqueous layer. Filter the benzene layer through a 10-mm. layer of anhydrous sodium sulfate. Wash the drying agent with more benzene to give 8.5 ml. of filtrate. Add 5 ml. of ethanol and 1.5 ml. of the tetraethylammonium hydroxide reagent. Mix, and read at 420 m μ .

NITROPHENIDE, *m,m'*-DINITRODIPHENYLDISULFIDE

Nitrophenide is reduced with sodium hydrosulfite to form an amino compound, which is diazotized and coupled with *N'*-1-naphthylenediamine

¹⁰⁵ E. J. Skerrett and E. A. Baker, *Analyst* **87**, 493-4 (1962).

dihydrochloride and read.¹⁰⁶ The interference of arsanilic acid is eliminated by diazotization in citric acid solution and reduction of the diazo compound with sodium hydrosulfite in phosphate buffer solution. Alternatively, if a solution of nitrophenide containing arsanilic acid is acidified and heated for 5 minutes, the coupling ability of arsanilic acid is destroyed. The nitrophenide is extracted from a feed sample with hot acetone, an aliquot is evaporated, and the nitrophenide is reduced in a phosphate buffer.

Procedure—Feed. To prepare the buffer for pH 6.6, dissolve 41.29 grams of anhydrous dibasic sodium phosphate in water and dilute to 1 liter. Dissolve 11.47 grams of citric acid monohydrate in water and dilute to 1 liter. Mix. Grind the sample, and to a 2-gram portion, add 0.5 gram of sodium hydrosulfite and 50 ml. of the buffer solution. Heat at 100° for 20 minutes. Slowly add 10 ml. of concentrated hydrochloric acid and heat for 5 minutes at 100° to destroy arsanilic acid and to precipitate colloidal sulfur. Connect to a compressed air or vacuum manifold and aerate vigorously for 15 minutes. Cool, dilute to 100 ml. with water, and mix. Filter, and discard the first 15 ml. of filtrate if turbid.

To a 5-ml. portion of the clear filtrate, add 2 ml. of a freshly prepared 0.1% sodium nitrite solution. After 5 minutes, add 2 ml. of 0.5% ammonium sulfamate solution and let stand for 2 minutes. Add 1 ml. of 0.1% N-1-naphthylethylenediamine dihydrochloride solution, mix, and let stand for 10 minutes. Dilute with 15 ml. of water and read at 545 m μ against water. Prepare another 5-ml. portion of the clear filtrate in the same way, omitting the coupling agent, and read against water. Subtract the absorbance of this blank from the sample absorbance.

TRINITROTOLUENE

Trinitrotoluene present as an impurity in dinitrotoluene is read in ammoniacal solution.¹⁰⁷

Procedure—Dissolve a sample of 1-5 ml. of dinitrotoluene, selected according to purity, in 10 ml. of acetone. Dilute to 15 ml. with ethanol and add 1.5 ml. of concentrated ammonium hydroxide. Digest at 60-65° for 30 minutes and cool. Dilute to 50 ml. with ethanol. Read the absorption curve over the range of 400-700 m μ .

¹⁰⁶ Richard T. Merwin, *J. Assoc. Offic. Agr. Chemists* **39**, 307-13 (1956).

¹⁰⁷ M. Laclercq and P. Agogu , *M m. Poudres* **44**, 7-13 (1962).

POLYNITROAROMATIC COMPOUNDS

Various polynitroaromatic compounds give a readable color with fluorene.¹⁰⁸ The color with *m*-dinitrobenzene fades too rapidly for reading. That from *o*-dinitrobenzene fades slowly; that from *p*-dinitrobenzene somewhat more rapidly. Other polynuclear hydrocarbons containing the cyclopentadiene moiety are potential reagents.

Procedure—To 5 ml. of sample in dimethylformamide, add 5 ml. of 0.1% fluorene in the same solvent. Add 1 ml. of 10% aqueous tetraethylammonium hydroxide. Dilute to 50 ml. with dimethylformamide. Read within 3 minutes. The wave length of maximum absorption is *o*-dinitrobenzene 695 m μ , *p*-dinitrobenzene 705 m μ , 1,4-dinitronaphthalene 738 m μ , 1,6-dinitropyrene 655 m μ , 6,12-dinitrochrysene 560 m μ . 3-Nitroazobenzene so treated absorbs at 665 m μ . The derivative of 1,3-bis-phenyl-azobenzene absorbs at 665 m μ , but requires 15-20 minutes to develop the color.

HEXANITRODIPHENYLAMINE

At pH 4, hexanitrodiphenylamine forms a deep orange anion.¹⁰⁹ Other nitro compounds do not interfere. The solution conforms to Beer's law for 2-14 micrograms per ml. of the final dilution.

Procedure—Mix an appropriate aqueous sample with 6 ml. of 70% potassium citrate solution and dilute to 50 ml. Read at 418 m μ .

¹⁰⁸ E. Sawicki and T. W. Stanley, *Anal. Chim. Acta* **23**, 551-6 (1960).

¹⁰⁹ R. D. Tiwari and J. P. Sharma, *Talanta* **10**, 933-4 (1963).

CHAPTER 2

ALIPHATIC AMINES AND AMIDES

SOME MULTIFUNCTIONAL amines and amides appear in Volume IIIA. They include, among the simpler compounds, *p*-anthranilic acid (p. 392), *p*-aminobenzoic acid (p. 394), *p*-aminosalicylic acid (p. 395), acetamide (p. 316), *N*-butylacetanilide (p. 381), and diaminodiphenylsulfone (p. 476). Thiacetazone, which is 4'-formylacetanilide thiosemicarbazone, is on page 472; amethopterin, which is methotrexate, 4-amino-*N*¹⁰-methylpteroryl-glutamic acid, is on page 372; amiphenazole, which is fenamizole, 2,4-diamino-5-phenylthiazole, is on page 465. For 1,2-bis(methylthio)ethane, see page 454. Benzalkonium chloride appears on page 492, Volume IIIA, as a halogen compound, and also in this chapter as a quaternary. *d*-Glucosamine appears in Volume IIIA, on pages 172-4, and in this chapter. Conditions for determination of acetamide, fluoroacetamide, and *N*-methylacetamide as hydroxamic acids are shown in Volume IIIA, on pages 314-6. Epinephrine, adrenaline, appears in Volume IIIA, pages 130-9. However, it is repeated here as catecholamines. In this volume, a reaction of pyrocatechol with dibutylamine in acetone is shown also applicable to diethylamine and to diethanolamine, on page 137. For adenine, 6-aminopurine, see Chapter 6, page 567. Procaine appears in this chapter, and in Chapter 5, on page 404. A method for nitramines appears in Chapter 1, page 12. For pyrazinamide, pyrazine carboxamide, by sodium nitritopentacyanoferroate, see page 496. For 3-methyl-2-benzothiazalone hydrazone, see page 551. For iproniazid, 1-isonicotinic-2-isopropylhydrazide, and nialamide, *N*-benzyl- β -(isonicotinylhydrazino) propinonamide, see page 494.

p-Hydroxyphenylmethylaminoethanol, synephrine, at pH 1 when excited by light of 270 m μ , fluoresces at 310 m μ . Propylene diamine complexes with cupric ion, but at least 2 moles of the former must be present to stabilize the maximum absorption at about 530 m μ .¹ Amides and nitriles are determined as hydroxamic acids,² in some cases, with propylene glycol as solvent.³

¹ James L. Hall and Thomas J. Carpenter, *Proc. W. Va. Acad. Sci.* 25, 37-9 (1953).

² J. G. Polya and P. L. Tarden, *Anal. Chem.* 23, 1036-7 (1951).

³ Saul Soloway and Abraham Lipschitz, *Anal. Chem.* 24, 898-900 (1952).

Treatment of 1,1'-dianthrime in concentrated sulfuric acid with a large excess of boric acid at 120° yields a 1:1 complex in a few minutes.⁴ Increasing moisture content shifts the color toward shorter wave lengths. The strongest absorbance is obtained in the presence of 10% water at 630 m μ . This corresponds with Beer's law, and expected impurities do not interfere. *Crystal violet base* or *hexamethyl-4-rosaniline*, is *tri-amino-diphenyl-tolylcarbinol*. It is read in benzene in the form of its picrate.⁵

Procaine amide, *p-amino-N-(2-ethylaminoethyl) benzamide* is read as the Schiff base formed with vanillin in an acid medium.⁶ The same reaction is given by *nadisan*, *etazol*, and *sulfanilylurea*. *Sympathol* is determined by Millon's reagent.⁷

Differential absorption at two wave lengths in the ultraviolet is effective in reading 2(*diphenylmethoxy*)*ethyl*dimethylamine *hydrochloride*⁸ in mixtures. This is also applicable to *N-m-bromobenzyl-N'-N'-diethyl-N-1-pyridylethylenediamine maleate*. *Glucuronamide* and *glucuronolactone* are determined in mixtures by the difference in their rate of reaction with hydroxylamine to form hydroxamic acids.⁹ The color is then developed by ferric ion. At pH 1, *hydroxyamphetamine* activated at 275 m μ fluoresces at 300 m μ .¹⁰

Acridine is a mixture of 2,8-diamino-10-methylacridinium chloride and 2,8-diaminoacridine. Buffered at pH 6.0, it is read fluorimetrically to 0.01 mg.%.¹¹ *Chloropropham* is extracted from white potatoes by macerating with dichloromethane and centrifugation. After passage through an alumina column, the extract is evaporated to dryness. The residue taken up in carbon bisulfide is read at 1210 and 1110 cm.¹² The cupric complex of *diethylenetriamine* absorbs at 620 m μ ; that of *ethylene-diamine*, at 550 m μ . The difference in wave lengths permits determination of more than 0.1% of the former in the latter.¹³

α -Aminocaproic acid and α,ϵ -diaminocaproic acid are determined with

⁴ Eitaro Hongo, *Bunseki Kagaku* **10**, 1200-3 (1961).

⁵ M. Rebek and M. K. Semlitsch, *Monatsh. Chem.* **92**, 254-60 (1961).

⁶ G. I. Luk'yanchikova and V. N. Bernshtein, USSR Patent 141,486, Oct. 16, 1961.

⁷ K. Agrawal and K. Steiger-Trippi, *J. Mond. Pharm.* **3**, 124-39 (1960).

⁸ G. Machek and F. Lorenz, *Sci. Pharm.* **31**, 17-26 (1963).

⁹ Tsukinaka Yamanaka, Shinichi Asai, and Jiro Aoki, *Arch. Pract. Pharm., Japan* **22**, 60-63 (1962).

¹⁰ Sidney Udenfriend, Daniel E. Duggan, Bruno M. Vasta and Bernard B. Brodie, *J. Pharmacol. Exptl. Therap.* **120**, 26-32 (1957).

¹¹ T. Bičan-Fišter, *Acta Pharm. Jugoslav.* **10**, 161-6 (1960).

¹² C. E. Ferguson, Jr., L. N. Gard, R. H. Shupe, and K. S. Dress, *J. Agr. Food Chem.* **11**, 428-31 (1963).

¹³ P. Kuba and T. Banič, *Chem. Zvesti* **17**, 445-8 (1963).

ninhydrin at pH 1.0 and 6.4, respectively, and by differential colorimetry.¹⁴ To read β -phenylisopropylhydrazine, iproniazid, or nialamide in the ultraviolet, dissolve in water, 1:200 hydrochloric acid, isopropanol, or ethanol. To read in the visible range, dissolve in glycol or acetone.¹⁵ Prothiadene, 11-(β -dimethylaminopropylidene)-6-hydrodibenzo[β , ϵ] thi-epin in ethanol is read at 233, 263, and 305 m μ .¹⁶

Metanephrine (3-o-methyladrenaline) and normetanephrine (3-o-methylnoradrenaline) are determinable by fluorescence.¹⁷ Collect a 24-hour specimen of urine with addition of 3.5 grams of sodium sulfite and 1.5 gram of sodium fluoride. Adjust an aliquot to pH 1, dilute appropriately, and heat at 100° for 1 hour. Adjust the pH to 8.5 with sodium hydroxide solution. Pass through a column of alumina to remove adrenaline and noradrenaline. Adjust the percolate to pH 6.2 and sorb the metanephrine and normetanephrine on 100-200 mesh Amberlite CG-50. Elute with 1:35 acetic acid. Follow technics for epinephrine thereafter.

2-Ethoxybenzamide is read in chloroform at 293 m μ .¹⁸ Sevin, 1-naphthyl methylcarbamate, is extracted with methanol for reading at 280 m μ .¹⁹ The proportionality is satisfactory up to 30 ppm. in the final solution. 6-Aminopenicillanic acid is determined by the reaction with *p*-dimethylaminobenzaldehyde.²⁰ The method is applied to the Auto-Analyzer at 415 m μ .

Poldine methosulfate, which is (1-methyl-pyrrolidiny)l)methylbenzilate methyl methosulfate, forms a blue complex with ammonium cobaltothiocyanate.²¹ This is extractable with chloroform to read at 322 m μ . It can be determined in the presence of 1-5 times as much of its decomposition products. When nialamide, *N*-isonicotinyl-*N'*-[β -(*N*-benzylcarboxamidovethyl)hydrazine, is treated with ammonium molybdate at pH 3 in oxalic acid solution, the intense blood red is stable for 24 hours.²²

Amines in boiler-feed water are determined at the 0-10-ppm. level

¹⁴ K. Czerepko and N. Wolosowicz, *Talanta* **10**, 813-15 (1963).

¹⁵ H. V. Tapia, *Anales. Fac. Quím. Farm. Univ. Chile* **14**, 88-97 (1962).

¹⁶ F. Jančík and B. Kakáč, *Českoslov. farm.* **13**, 3-6 (1964).

¹⁷ S. Brunjel, D. Wybenga and V. J. Johns, Jr., *Clin. Chem.* **10**, 1-12 (1964).

¹⁸ H. Siedlanowska, *Farm. Polska* **20**, 160-3 (1964).

¹⁹ Jan Kanazawa, Tetuki Kawahara, and Rokuro Sato, *Japan Analyst* **10**, 906-8 (1961).

²⁰ Joseph Bomstein and William G. Evans, *Anal. Chem.* **37**, 576-8 (1965).

²¹ D. O. Singleton and G. M. Wells, *J. Pharm. Pharmacol.* **12**, Suppl. 171T-175T (1960).

²² G. Sandri Cavicchi and M. P. Quaglio, *Boll. chim-farm.* **103**, 660-4 (1964).

by *m*-cresol purple, bromophenol blue, bromocresol green, phenol red, or xylenol blue.²³ The reagent is 0.1 gram of indicator in 10 ml. of denatured alcohol diluted with water. Then 0.5 ml. of concentrated sulfuric acid is added before dilution to 100 ml. The 5-ml. sample plus 1 ml. of indicator is diluted to 10 ml., and the amine color extracted with a chlorinated solvent.

To determine *succinylcholine chloride*, it is separated from succinylmonocholine chloride and choline chloride on a strong acid-type cation exchange resin. It is converted to the hydroxamic acid with hydroxylamine. As the ferric complex, this is read at 540 m μ .²⁴ The same method is applicable to the metabolites. For determination of *diethylenetriamine-penta-acetic acid* and *diaminobutanetetraacetic acid*, follow the procedure for EDTA by ferric sulfosalicylate.

Imipramine is read by the reineckate reaction, or photometrically, with congo red or methyl orange.²⁵ It gives an intense purple when activated at 253.7 m μ . The maximum absorption in 1:200 hydrochloric acid is at 250.5 m μ , with linear absorption up to 4 mg. per 100 ml. It is determined in alkaline tissue extracts by extraction with ether. Thereafter, it is extracted into 1:200 hydrochloric acid for reading.²⁶ For *neostigmine bromide* in injection solutions, mix 5 ml. of sample with 2 ml. of 1:1750 sulfuric acid, dilute to 10 ml. and read at 261 m μ .²⁷ Similarly, read at 260 m μ for *neostigmine methylsulfate*, or at 266 m μ for the sum of the two.

For *glutamine* and *asparagine* in blood samples of 0.01-0.1 ml., an enzyme is added that exhibits glutaminase and asparaginase activity. Such an enzyme may be prepared from *Pseudomonas* GG13. Glutamine and asparagine are converted to ammonia and absorbed on a glass rod wet with 1:35 sulfuric acid, which is suspended in the incubation vessel. The ammonia is then determined by sodium phenoxide, sodium nitroprusside, sodium carbonate, and sodium hypochlorite.²⁸ For glutamine by hydroxylamine and ferric chloride in the presence of glutathione, glutamic acid, and γ -aminobutyric acid, see page 273. To determine *2-amino-2-*

²³ A. S. Pearce and E. L. Streatfield, German Patent 1,190,229.

²⁴ Hisashi Nogami and Tokuji Suzuki, *Chem. Pharm. Bull.* (Tokyo) 9, 646-50 (1961).

²⁵ E. Domagalina and L. Pyzyborowski, *Chem. Anal. Warsaw* 7, 1153-65 (1962).

²⁶ S. Denton, *Analyst* 87, 234-6 (1962).

²⁷ J. Kraemarova and J. Kraemar, *Ceskosl. Farm.* 13, 324-31 (1964).

²⁸ Mohyi El-Din A. Ramadan and D. M. Greenberg, *Anal. Biochem.* 6, 144-52 (1963).

desoxyhexane and *2-deoxygalactose*,²⁹ mix 1 ml. of solution and 7 ml. of concentrated sulfuric acid. Heat for 10 minutes and read at 325 m μ .

Aminophylline has absorption maxima at pH 9.5 at 243.5 m μ with a value for $E_{1\text{ cm.}}^{1\%}$ of 170 and at 273.5 with $E_{1\text{ cm.}}^{1\%}$ of 500. It conforms to Beer's law over the range of 0.5-1.2 mg%.³⁰ When an aqueous solution of *theophylline* is heated with an equal volume of 40% sodium hydroxide solution at 120° for 15 minutes, it is probably converted to theophyllidine. Thereafter, it is acidified with acetic acid. When coupled with azobenzene-*p*-sulfonic acid, it is read at 482 m μ . Alternatively, it is coupled with diazotized *p*-nitroaniline and read at 496 m μ . Free theophylline is extractable from pharmaceutical products with ether. Combined theophylline remains in the aqueous layer and is determined as above. Caffeine, salicylamide, and phenacetic interfere.

Bufotenin, 3-(2-dimethylaminoethyl)-5-indolol is determined fluorometrically.³¹ It is precipitated with 2,4-dinitrophenyl hydrazine to separate it from carbonyl compounds. Treatment with carbon bisulfide then forms *N*-substituted dithiocarbamic acids. Products of primary and secondary amines are extracted with aqueous alkali, leaving the tertiary amine in the organic phase.

ALIPHATIC AMINES

Cobalt thiocyanate forms a complex with primary, secondary, or tertiary amines, which is extracted at pH 1.4 with pentyl alcohol.³² Kerosene is added to aid solution. Ferric iron is removed by complexing with sodium dihydrogen orthophosphate. As little as 5 ppm. of amine in aqueous solution can be determined. Beer's law is followed up to 200 ppm. of amine.

When the aliphatic amine is added to an alcoholic solution containing excess cupric chloride, a yellow complex is formed.³³ As much as 13 mg. of water per ml. can be present without interfering. If larger amounts of water are present, the sample is diluted with ethanol or extracted with chloroform. Chloroform extraction also eliminates interference from sodium and potassium hydroxide. Acids are neutralized with potassium

²⁹ J. D. Cipera, *Analyst* **85**, 517-19 (1960).

³⁰ Maria Amelia Andrade and Maria Mannela Leite Lanacio, *Rev. port. farm.* **10**, 141-5 (1960).

³¹ H. Gross and F. Franzen, *Biochem. Z.* **340**, 403-12 (1964).

³² P. J. Lloyd and A. D. Carr, *Analyst* **86**, 335-8 (1961).

³³ Herbert M. Hershenson and David N. Hume, *Anal. Chem.* **29**, 16-19 (1957).

carbonate. Sodium acetate does not interfere, but sodium benzoate does. Beer's law is followed.

Primary, secondary, and tertiary amines may be measured by reaction with picric acid.³⁴ (Cf. Vol. III, p. 31.) Amines boiling below 80° cannot be determined by this method. The sample is extracted with chloroform and distilled over with this solvent.

Fatty amines on potassium salts are developed with anhydrobisindandione in diethyl ether.³⁵ Up to 0.02 mg. of C₁₄, C₁₆ or C₁₈ fatty amine per gram of potassium salt can be determined. In general, amines in aqueous solution react with such sulfonephthaleins as bromophenol blue. These are extractable with a small volume of chloroform, usually 10% or less, to determine amounts in the low gamma range.³⁶ Bromocresol green³⁷ and bromocresol purple³⁸ are similarly used.

Primary amines are read at 2023 m μ and secondary amines at 1538 m μ in the presence of tertiary amines.³⁹ A suitable concentration is 1-25%. Amides, nitriles, alcohols, and esters up to 10% do not interfere seriously. Aliphatic aldehydes interfere by reacting with the primary and secondary amines.

Diphenylpicrylhydrazyl is an intensely violet, free radical. While it is primarily applicable to aliphatic amines, it has some reactions with the aromatics. The reaction is to extract a hydrogen atom to form the yellow diphenylpicrylhydrazine. The reading is by decrease of the violet color.⁴⁰ Since the rate of reaction differs for each amine, the comparison must be against a standard curve developed with the same amine. Often, one amine can be determined in the presence of another due to differences in their rate of reaction. The rates of reaction of typical aliphatic amines are shown in Chapter 5, Table 23, and the procedure, on page 378.

Lower aliphatic amines in biological material react with 1-chloro-2,4-dinitrobenzene to form yellow *N*-alkylanilines.⁴¹ Dimethylamine, diethylamine, and their derivatives, di-*n*-propylamine and di-*n*-butylamine, react with the reagent. Diisopropylamine and diisobutylamine and adren-

³⁴ Yuji Takayama, *Bunseki Kagaku* **6**, 306-8 (1957).

³⁵ A. Singewald, *Z. anal. Chem.* **164**, 219-32 (1958).

³⁶ A. Mukerjee and P. Mukerjee, *J. Appl. Chem.* (London) **12**, 127-9 (1962).

³⁷ Masaru Aoki, Yoji Iwayama and Noboru Yata, *Yakugaku Zasshi* **82**, 918-21 (1962).

³⁸ L. I. Nemtseva, A. D. Semenov and V. G. Datsko, *Zhur. Anal. Khim.* **19** 383-5 (1964).

³⁹ Fred H. Lohman and William E. Norteman, Jr., *Anal. Chem.* **35**, 707-11 (1963).

⁴⁰ G. J. Papariello and M. A. M. Janish, *Anal. Chem.* **37**, 899-902 (1965).

⁴¹ L. Ekladius and H. K. King, *Biochem. J.* **65**, 128-31 (1956).

aline do not. The reagent itself gives appreciable absorption at 358 $m\mu$. The maximum absorption wave length and corresponding extinction coefficients for some 2,4-dinitroanilines are given in Table 7. Beer's law is followed up to 0.3 mg. of amine nitrogen.

TABLE 7. PROPERTIES OF 2,4-DINITROANILINES $[RR'.N.C_6H_3(NO_2)_2]$

R	R'	$\lambda_{\max.}$ ($m\mu$)	$E_{1\text{cm.}}^{1\%}$	ϵ
$n\text{-C}_3\text{H}_7\text{-}$	H	358	827	18 700
$n\text{-C}_4\text{H}_9\text{-}$	H	358	725	17 400
$(\text{CH}_3)_2\cdot\text{CH}\cdot\text{CH}_2\text{-}$	H	358	777	18 600
$(\text{CH}_3)_2\cdot\text{CH}\cdot\text{CH}_2\cdot\text{CH}_2\text{-}$	H	358	695	18 200
$\text{C}_6\text{H}_5\cdot\text{CH}_2\cdot\text{CH}_2\text{-}$	H	348	686	19 100
$\text{CH}_3\text{-}$	$\text{CH}_3\text{-}$	370	900	19 000
$\text{C}_2\text{H}_5\text{-}$	$\text{C}_2\text{H}_5\text{-}$	370	900	21 500

Note: Extinction coefficients were measured at $\lambda_{\max.}$

Procedure—*By cobalt thiocyanate.* Uranium leach liquors containing less than 0.5 gram of ferric iron per liter. Adjust the pH of the sample to 1.4 with orthophosphoric acid. Add cobaltous nitrate and sodium thiocyanate to give a concentration of 40 grams of each per liter of solution and readjust the pH to 1.4. Add slightly more solid sodium dihydrogen orthophosphate than is necessary to completely discharge the color of the ferric thiocyanate before the amine complex is extracted. Extract the blue complex by shaking vigorously with 10 ml. of 20% pentyl alcohol solution in kerosene for 30 seconds. After 10 minutes, separate the aqueous layer. Filter the solvent layer and read the filtrate at 625 $m\mu$ against a reagent blank.

Samples containing more than 0.5 gram of ferric iron per liter. To a 30-ml. sample, add 5 ml. of 10% ascorbic acid solution to reduce the iron. Add 10 ml. of a solution containing 20% each of cobaltous nitrate and sodium thiocyanate. Add at least 10 grams of sodium dihydrogen orthophosphate. Add additional orthophosphate as needed to discharge the color of the ferric thiocyanate complex. Adjust the pH to 2.5 with orthophosphoric acid and follow the previous procedure, starting at "Extract the blue complex . . ."

By cupric chloride. General. Dilute the sample with absolute ethanol so that a 1-5 ml. aliquot contains 10-80 micromoles of amine. To 5 ml. of

0.8525% cupric chloride dihydrate solution in absolute ethanol, add 15 ml. of absolute ethanol. Add 1-5 ml. of sample solution and dilute to 25 ml. with absolute ethanol. Mix and let stand at room temperature for 20 minutes. Read at 860 $m\mu$ against a reagent blank.

Samples containing large amounts of water or inorganic bases. Dilute the sample to 5 ml. with water. If the sample is aqueous, take a 5-ml. aliquot. If the sample is alcoholic, dilute with water to reduce the alcohol content to 20% or less and take a 5-ml. aliquot. To the 5-ml. aliquot, add 1 gram of potassium carbonate and dissolve with shaking. Extract with 5 ml. of chloroform saturated with water by shaking with a 20% potassium carbonate solution. Shake for 1 minute and add 1 ml. of the chloroform extract to 5 ml. of 0.8525% cupric chloride dihydrate solution in absolute ethanol. Dilute to 25 ml., mix well, and let stand at room temperature for 20 minutes. Read at 860 $m\mu$ against a reagent blank.

By picric acid. To a 1-ml. sample containing 0.1-0.8% of amine, add 2 ml. of 30% sodium hydroxide solution and dilute to 20 ml. Shake a 1-ml. aliquot with 8 ml. of chloroform for 3 minutes and let separate for 3 minutes. Repeat the extraction twice with 8-ml. portions of chloroform. Distil the combined chloroform layers at the rate of 1 drop every 4 seconds until 1 ml. of distillate is collected. To the distillate, add 1 ml. of 0.2% picric acid solution in chloroform and dilute to 25 ml. with chloroform. Read after 1 minute at 415 $m\mu$ against water.

By anhydrobisindandione. Potassium salts. To a 5-gram sample, add 5 ml. of acid phosphate-citrate buffer for pH 6.5-7 (Vol. 1, p. 176) and 15 ml. of 0.02% anhydrobisindandione solution in diethyl ether. Dilute with water to 50 ml. Shake vigorously, allow the layers to separate, and read the ether layer at 520 $m\mu$.

By 1-chloro-2,4-dinitrobenzene. Place a sample of up to 10 ml., containing 0.05-0.025 mg. of amine nitrogen, in a micro-steam distillation apparatus. Add 2 ml. of propan-1-ol to the receiver and pass steam until 5 ml. of distillate is collected in 2 minutes. Add 1 ml. of 0.5% 1-chloro-2,4-dinitrobenzene solution in 50% propan-1-ol. Stopper, place in a cold water bath, and let stand at room temperature for 15 minutes. Gradually bring the bath to a boil over a period of 30 minutes and heat at 100° for 30 minutes. Cool, dilute to 10 ml. with 50% propan-1-ol and read against a blank at 450 $m\mu$. Read the blank against the solvent. To prepare the

blank, carry a standard containing 0.14 mg. of amine nitrogen in 5 ml. of 50% propan-1-ol through the procedure, omitting the steam distillation step.

By bromocresol green. Mix 2 ml. of neutral sample containing about 10^{-4} mole of the amine with 2 ml. of phosphate-citric acid buffer for pH 5.6. Add 1 ml. of 1% aqueous bromocresol green and 10 ml. of chloroform. Shake well, let separate, and isolate the chloroform layer. Shake with 0.1 gram of anhydrous sodium sulfate and let stand for 30 minutes. Mix 5 ml. of this clear layer with 5 ml. of 1:1 triethanolamine-ethanol. Read at 630 $m\mu$ against a reagent blank.

By bromocresol purple. Water. Steam-distil a 250-ml. sample, collecting 150 ml. of distillate in 15 ml. of 1:11 hydrochloric acid. If aromatic amines are also present, add 0.5 ml. of 1:350 sulfuric acid. Evaporate to dryness at 100°. Take up the residue in 3 ml. of citrate buffer for pH 3.5. Mix 2 ml. with 0.5 ml. of 0.1% solution of bromocresol purple. Shake for 2 minutes with 4 ml. of chloroform. Separate the chloroform layer and add 0.5 ml. more to clarify any haze. Read at 410 $m\mu$. The slope of the curve will depend on what amines are present. The method conforms to Beer's law for 0.2-6 micrograms of amino nitrogen.

PRIMARY ALIPHATIC AMINES

Primary aliphatic amines react with cupric chloride and salicylaldehyde in the presence of triethanolamine. The resulting copper salicylaldehydeimine is extracted into 1-hexanol and the copper in the 1-hexanol is determined by reaction with bis(2-hydroxyethyl)dithiocarbamic acid.⁴² Triethanolamine is added to shift the equilibrium of the reaction between cupric chloride and the imine. Aromatic amines, compounds containing more than one primary amine group, such as ethylenediamine and diethylenetriamine, and primary amines that are branched in the 2 position, such as tertiary and secondary butylamine and isopropylamine, generally do not react quantitatively. Exceptions are propylenediamine and 2-ethylhexylamine, which may be determined by this procedure.

There is interference by more than 0.01 mg. of ammonia, corresponding to an absorbance of 0.03. More than 0.5 gram of combined tertiary and secondary amines interfere by solubilizing the copper complex in the

⁴² Frank E. Critchfield and James B. Johnson, *Anal. Chem.* 28, 436-40 (1956).

aqueous layer. Strong oxidizing agents and reducing agents react with the reagent.

Primary aliphatic amines, when reacted with ninhydrin in the presence of pyridine, exhibit absorption curves with peaks at 415 and 575 $m\mu$ although the appearances and shape of the curves differ.⁴³ Secondary and tertiary amines, with the possible exception of proline, do not give the color reaction with ninhydrin under the conditions of the test procedure. Generally, primary amines attached directly to rings, amides, and secondary and tertiary amines do not give the color with ninhydrin. The reactions are summarized in Table 8.

Primary amines reduce the absorption of the cupric-ethylenediamine complex.⁴⁴ The complex is stable at pH 4-11. The added amine enters the complex, disrupting two carboxyl linkages to the central copper ion of each cell of the polymer. Secondary amines have the effect to a far lesser extent, and tertiary amines have no effect. Most foreign inorganic anions do not interfere. Cations complexing with ethylenediamine tetraacetate interfere, many to only a slight extent.

Salicylaldehyde forms a yellow color upon reaction with primary amines.⁴⁵ Unsubstituted amides, *N*-alkylacetamide, and nitriles do not interfere. Secondary and tertiary amines give no color with salicylaldehyde in the presence of acetic acid. Beer's law is followed.

Primary aliphatic amines have a characteristic absorption band at 2023 $m\mu$.⁴⁶ Secondary amines are determined by an overtone. Amides, alcohols, nitriles, and esters up to 10% of the mixture do not interfere seriously.

Dibepirin, 8-(10,12-dioxodiindeno[3,2-b:2',3'-e]pyrid-11-yl)-1-naphthoyl chloride, is a reagent for primary amines.⁴⁷ The reagent is yellow but gives a blue-violet color with unsubstituted and monosubstituted amides in ethanolic alkaline solution. Esters and disubstituted amides do not give the color.

Primary amines react with ascorbic acid in dimethylformamide.⁴⁸ Secondary and tertiary amines do not interfere. The method is applicable to α -amino acids, with a minor revision of the procedure.

⁴³ Dean F. Davies, Kathryn M. Wolfe and H. Mitchell Perry, Jr., *J. Lab. Clin. Med.* **41**, 802-810 (1953).

⁴⁴ Irvin M. Citron and Allan Mills, *Anal. Chem.* **36**, 208-10 (1964).

⁴⁵ Albert J. Milun, *ibid.* **29**, 1502-4 (1957).

⁴⁶ F. H. Lohman and W. E. Norteman, Jr. *ibid.* **35**, 707-11 (1963).

⁴⁷ G. Ya. Dubur and G. Ya. Vanog, *Izv. Akad. Nauk Latv. SSR, Ser. Khim.* 25-S (1962).

⁴⁸ J. Bartos, *Ann. Pharm. Franc.* **22**, 383-5 (1964).

TABLE 8. REACTION OF NINHYDRIN WITH NITROGEN COMPOUNDS IN THE PRESENCE OF PYRIDINE

Reaction Rate	Type of Compounds ^a	Examples
Rapid	$\begin{array}{c} \text{H} \\ \\ \text{R}-\text{C}-\text{NH}_2 \\ \\ \text{H} \end{array}$	Ethylamine, butylamine, isoamylamine, hexylamine, heptylamine, decylamine, dodecylamine, tetradecylamine, octadecylamine, tyramine, tryptamine, histamine, arterenol, glucosamine, glutamine, 2-phenylethylamine. Exception: Methylamine.
	$\begin{array}{c} \text{H} \\ \\ \text{R}-\text{C}-\text{COOH} \\ \\ \text{NH}_2 \end{array}$	Glycine, alanine, leucine, methionine, phenylalanine, tryptophane, tyrosine, histidine, lysine, arginine, ornithine, citrulline, glutamic acid, aspartic acid.
Retarded	$\begin{array}{c} \text{H} \\ \\ \text{R}-\text{C}-\text{NH}_2 \\ \\ \text{R}' \end{array}$	1,3-dimethyl pentylamine; 1-methyl heptylamine; 1-methyl 2-(4-hydroxyphenyl) ethylamine; 1-butyl,2-hydroxy 2-phenylethylamine; 1-phenylethylamine, cyclohexylamine. Exception: Amino acids.
	$\begin{array}{c} \text{H} \\ \\ \text{R}-\text{C}-\text{C}-\text{NH}_2 \\ \quad \\ \text{O} \quad \text{H} \end{array}$	α -dimethyl amino, 2,4-dihydroxy acetophenone; α -amino 3,4-dihydroxyacetophenone.
	Others	Methylamine. Proline.
No reaction \Rightarrow $-\text{NH}_2$	$\begin{array}{c} \text{R}-\text{C}-\text{NH}_2 \\ \\ \text{O} \end{array}$	Aniline, <i>p</i> -bromoaniline, <i>p</i> -nitroaniline, adenine.
	$\begin{array}{c} \text{H} \\ \\ \text{R}-\text{N}-\text{R}' \\ \\ \text{R}''\text{NH}_2 \end{array}$	Urea, acetamide.
		Epinephrine, xanthine. Exception: Proline.
		Ammonia, hydroxylamine.

^a R and R' represent any organic substituents; R'' represents OH or H.

Procedure—*By copper-salicylaldehyde.* To prepare the copper-salicylaldehyde reagent, dilute 15 ml. of redistilled triethanolamine, 0.5 ml. of salicylaldehyde, and 0.25 gram of cupric chloride dihydrate to 100 ml. with water, and mix. Prepare 2% bis(2-hydroxyethyl)dithiocarbamic acid solution in carbon disulfide and 5% diethanolamine solution in methanol. Before use, mix equal volumes of these two components.

To 2 ml. of copper-salicylaldehyde reagent, add the sample containing

TABLE 9. REACTION CONDITIONS FOR
DETERMINATION OF PRIMARY AMINES
BY THE COPPER-SALICYLALDEHYDE METHOD

<i>Compound</i>	<i>Primary amine, mg., Maximum</i>	<i>Time, Minutes^a</i>
Aminoethylethanolamine	1.10	30 to 60
<i>N</i> -Aminoethylmorpholine	0.85	15 to 60
Amylamine	1.20	15 to 45
Butylamine	0.70	15 to 60
Ethanolamine	0.50	15 to 60
Ethylamine	0.53	15 to 60
2-Ethylhexylamine	1.40 ^b	15 to 60
Hexylamine	1.10	15 to 60
Isoamylamine	1.10	15 to 45
Isobutanolamine	0.60	60 to 120
Isobutylamine	0.90	15 to 60
Isopropanolamine	0.60	15 to 60
Methylamine	0.30	15 to 60
Propylamine	0.73	15 to 60
Propylenediamine	0.42	10 to 20 ^c

^a Reaction time at 20° to 30° C. unless otherwise specified.

^b Make dilutions using a 10% solution of methanol.

^c Perform reaction at 98° ± 2° C. Use 50-ml. glass-stoppered graduated cylinders; do not stopper during reaction.

the maximum amount of amine, as listed in Table 9. The sample must not contain more than 0.01 mg. of ammonia or 0.5 grams of secondary and tertiary amines. Dilute to 10 ml. with water, stopper, and mix well. Let stand for the requisite time, as shown in Table 9. Dilute with 1-hexanol to 25 ml. Stopper, and shake vigorously 15-20 times. Allow the layers to separate.

Add 5 ml. of the hexanol layer to 5 ml. of *bis*(2-hydroxyethyl)dithiocarbamic acid reagent dropwise. Dilute to 25 ml. with methanol, stopper, and mix. Read against a reagent blank at 430 m μ .

By ninhydrin. Dilute a sample with a 1:99 mixture of concentrated hydrochloric acid and isopropanol so that the amine concentration is $2\text{--}20 \times 10^{-4}$ millimoles per ml. To a 1-ml. aliquot, add 5 ml. of pyridine and 2 ml. of 0.2% ninhydrin solution in isopropanol. Dilute to 10 ml. with 1:99 hydrochloric acid-isopropanol mixture. Mix, and heat at 85° for 7 minutes. Cool immediately in water and read at 575 m μ .

By salicylaldehyde. Amine acetates. To prepare the acetic acid reagent, dilute 4 ml. of glacial acetic acid to 200 ml. with chloroform. Dissolve a sample containing up to 1.5 millimoles of primary amine acetate in 17 ml. of chloroform. Add 3 ml. of acetic acid reagent, washing down the sides of the container during addition. Swirl gently. Add 5 ml. of 5% salicylaldehyde solution in chloroform and swirl gently. Heat at 30° for 100 minutes. Dilute with chloroform to 490 ml. and heat at 30° for 10 minutes. Dilute to 500 ml. with chloroform. Mix, and read after 5 minutes at 410 m μ against chloroform.

Primary amines. Follow the procedure for primary amine acetates, substituting 14 ml. of chloroform for 17 ml., and 6 ml. of acetic acid solution for 3 ml.

By ethylenediaminetetraacetate. Prepare a 2.497% solution of copper sulfate pentahydrate and a 3.84% solution of sodium ethylenediaminetetraacetate. To 4 ml. of each, add 0.5 ml. of sample containing about 0.15 *M* of unknown amine. Dilute to 25 ml. and read at 720 m μ . The various amines in the sample should be known for production of a curve. Thus primary diamines and polyamines not only lower the absorption of the complex but lower the wave length of maximum absorption.

By dibeprin. Dissolve a sample containing about 0.5 mg. of test substance in dioxan and add potassium carbonate. Boil for 30 seconds and add a few drops of 5% aqueous sodium hydroxide solution and a few drops of ethanol. The blue-violet color from primary amines reverts to yellow on acidification. The greatest sensitivity is with primary amines and aralkylamines.

By ascorbic acid. As a reagent, dissolve 0.05 gram of ascorbic acid in 0.5 ml. of water and dilute to 50 ml. with dimethylformamide. To a sample of 0.01-0.1 mg. in dimethylformamide, add 2 ml. of reagent and dilute to 5 ml. with dimethylformamide. Heat to 100° for 10 minutes and cool to 15-20°. Read at 390 or 525 m μ .

SECONDARY AMINES

Secondary aliphatic amines can be determined in the presence of primary amines, tertiary amines and ammonia by sodium nitroprusside, and acetaldehyde with formation of a violet-blue color.⁴⁹ The optimum pH is 9.6-10.2.

⁴⁹ C. F. Cullis and D. J. Waddington, *Anal. Chim. Acta* **15**, 158-63 (1956).

Secondary amines with the nitrogen atom attached to a CH group are difficult to acetylate and show low reactivity by this method. Beer's law is followed for dimethylamine at 565 $m\mu$ for concentrations up to 3×10^{-3} mole $^{-1}$; for diethylamine at 565 $m\mu$ for concentrations up to 8×10^{-3} mole $^{-1}$; for diisobutylamine at 590 $m\mu$ for concentrations up to 3×10^{-3} mole $^{-1}$; and for di-*n*-propylamine, di-*n*-butylamine, and di-*n*-amylamine at 580 $m\mu$. Diisopropylamine and di-*sec*-butylamine do not give color with the reagents. Since the colored compound is unstable, a timed interval for reading is required. Ammonia, methylamine and trimethylamine do not interfere.

Bromocresol green in isopropanol gives a blue-green color with secondary amines.⁵⁰ Primary amines are removed by reaction with salicylaldehyde in isopropanol. Sodium hydroxide is added to bromocresol green reagent to neutralize the bromocresol green and prevent its neutralization of the secondary amine. Less than 1% of tertiary amine does not interfere.

With carbon disulfide, secondary amines form dithiocarbamic acid which reacts with the cupric ion to form a yellow salt. This is read as a measure of the amount of secondary amine present. Both aliphatic and aromatic secondary amines may be analyzed by this procedure.⁵¹ Tertiary amines do not interfere.

Primary amines react with the reagent but give lower color intensities, which usually result in an error of 1% or less in determination of secondary amines; but over 0.1 mg. of primary amine per sample cannot be tolerated. Pyridine, triethylamine, tributylamine, *N,N*-dimethylaniline, 1,3-diphenylguanidine, diphenylamine, 3-carbonyl indoles, and ammonia do not react with the reagents. Secondary amines do not react if they are highly conjugated. Beer's law is followed for 0.01-0.09 mg. of diethylamine and 0.03-0.1 mg. of *N*-methylaniline.

As a variant on this technic, the nickel dithiocarbamates of the amines are precipitated from benzene solution.⁵² When treated with an alkaline solution, the primary and tertiary amine derivatives are dissolved from the precipitate. The nickel of the secondary amine derivative is replaced by copper and read at 435 $m\mu$.

In general, aliphatic secondary amines are reacted in acid solution at 50° with nitrous acid for 1-2 minutes to form nitroso compounds. The rapid reaction reduces interference by primary and tertiary amines. The

⁵⁰ A. J. Milun and J. P. Nelson, *Anal. Chem.* **31**, 1655-7 (1959).

⁵¹ Gerald R. Umbreit, *ibid.* **33**, 1572-3 (1961).

⁵² Luisa Nebbia and Franco Guerrieri, *Chimica e industria* **35**, 896-9 (1953).

solution is made strongly alkaline with sodium hydroxide solution, extracted with hexane, and read at 235 $m\mu$. They conform to Beer's law over the range of $5.1 \cdot 10^{-6}$ – $5.1 \cdot 10^{-4}$ M in the hexane.⁵³ The same curve is applicable for dipropylamine and for dihexylamine. Diethylamine deviates somewhat. The method is not applicable to dimethylamine.

The amides of secondary amines with 3,5-dinitrobenzoyl chloride form blue colors with acetone and sodium ethylate.⁵⁴ The reaction is applicable to diethylamine, phenylethylamine, phenylbutylamine, and piperidine.

For a specific method for dimethylamine in hydrocarbons, see dimethylformamide, page 91.

Procedure—*By sodium nitroprusside and acetaldehyde.* Prepare a reagent solution containing 1% of sodium nitroprusside and 10% of acetaldehyde. Add 5 ml. of the reagent to 20 ml. of a buffer containing 0.953% of borax and 0.53% of sodium carbonate. Add 5 ml. of water and 5 ml. of sample and read at a timed interval. The wave length is 565 $m\mu$ for dimethylamine and diethylamine, 580 $m\mu$ for di-*n*-propylamine, di-*n*-butylamine and di-*n*-amylamine, and 590 $m\mu$ for diisobutylamine.

By bromocresol green in primary amines. To prepare the salicylaldehyde reagent, dilute 10 ml. to 100 ml. with isopropanol, distil, and discard the first 10 ml. To prepare the bromocresol green reagent, mix 0.03 gram with 10 ml. of isopropanol and add 0.4 ml. of 0.4% sodium hydroxide solution. Dilute to 200 ml. with isopropanol.

Dissolve a 0.625-gram sample of dodecylamine or octadecylamine in 25 ml. of isopropanol. To a 2-ml. aliquot, add 2 ml. of salicylaldehyde reagent. Stopper, swirl, and let stand for 10–30 minutes. Add 2 ml. of bromocresol green reagent and dilute to 10 ml. with isopropanol. Shake thoroughly. After 5 minutes, read at 627 $m\mu$ against isopropanol.

As dithiocarbamic acid. Strongly acidic samples should be neutralized with sodium hydroxide or ammonia before analysis. To prepare the reagent, mix 35 ml. of carbon disulfide, 25 ml. of pyridine, and 65 ml. of isopropanol. To prepare cupric chloride reagent, dissolve 0.1 gram of cupric chloride dihydrate in 250 ml. of water, and dilute to 500 ml. with pyridine.

To a 1-ml. sample, add 4 ml. of carbon disulfide reagent and 2 ml.

⁵³ S. J. Clark and D. J. Morgan, *Mikrochim. Acta* **1956**, 966–76; D. J. Morgan, *ibid.* **1958**, 104–10.

⁵⁴ Hermann Kurz and Gerhard Renner, *Z. anal. Chem.* **186**, 369–72 (1962).

of cupric chloride reagent. Agitate and let stand for 5-20 minutes. Add 3 ml. of 10% acetic acid and 3 ml. of benzene. Invert several times and allow the layers to separate. Dilute 4 ml. of the upper organic phase to 5 ml. with isopropanol. Read according to Table 10.

TABLE 10. REACTION OF COMPOUNDS
AS DITHIOCARBAMIC ACIDS

Compound	Wave Length of Absorption Max., $m\mu$	Time to Reach Max. Absorbance, Min. $\epsilon' \times 10^2$ ^a	
Piperidine	440	30	5.37
Di- <i>n</i> -butylamine	440	30	7.08
<i>N</i> -Methylaniline	445	20	5.36
Diethylamine	440	60	9.67
Isoleucine	360		
<i>n</i> -Butylamine	350, 430 ^b		
Aniline	355, 430 ^b		
Neomycin B	390		

^a Based on concentration in original sample aliquot.

^b Secondary amine impurity is suspected.

PRIMARY AND SECONDARY AMINES

A method developed for deoxyephedrine is applicable to primary and secondary amines.⁵⁵ The amine is treated with 1-fluoro-2,4-dinitrobenzene and excess reagent hydrolyzed to 2,4-dinitrophenol. The 2,4-dinitrophenylamine is extracted with cyclohexane or tetrachlorethane and read at wave lengths shown in Volume IV, page 30.

The method has been applied to plant amines separated by ion-exchange chromatography.⁵⁶ The maxima are shown in Table 11. There is no interference by glycine, L-valine, L-alanine, L-lysine, L-leucine, DL-serine, L-threonine, DL-glutamic acid, L-arginine, DL-methionine, L-proline, L-histidine, or L-ornithine in 100 microgram amounts because the 2,4-dinitrophenyl derivatives are insoluble in tetrachlorethane. DL-phenylalanine and L-tyrosine interfere.

Primary and secondary amines react with 4'-nitroazobenzene-4-car-

⁵⁵ Floyd C. McIntire, Lois M. Clements and Muriel Sproull, *Anal. Chem.* 25, 1757-8 (1953).

⁵⁶ M. Richardson, *Nature* 107, 290-1 (1963).

TABLE 11. MAXIMA OF AMINES
AS 2,4-DINITRO DERIVATIVES

<i>Amine</i>	<i>mμ</i>
Ammonia	335
Methylamine	352.5
Ethylamine	352.5
Ethanolamine	352.5
Isoamylamine	352.5
Isobutylamine	352.5
<i>p</i> -Phenylethylamine	352.5
Dimethylamine	377.5
Diethylamine	385
<i>N</i> -Methylethanolamine	377.5
Putrescine	355
Cadavarine	355

boxylic acid chlorides to give derivatives separable by paper chromatography.⁵⁷ When the technic is applied to blood,⁵⁸ recovery is good except for histamine. Corresponding experiments with urine give good recoveries except for β -phenylethylamine, ethanolamine, tyramine, tryptamine, and histamine.

It is also possible to read the reaction product in the presence of excess reagent. Then the optimum absorbance at 365 $m\mu$ gives too high a blank and reading must be at a longer wave length. For an example, see the determination of nylon amines, page 212. Primary and secondary amines are determinable by *p*-nitrophenylazobenzoyl chloride.⁵⁹

Procedure—By 1-fluoro-2',4-dinitrobenzene. *Blood*. Mix 2 ml. of blood with 12 ml. of water. Add 1 ml. of 20% zinc sulfate heptahydrate solution and mix. Add 1 ml. of 4% sodium hydroxide solution and mix. Centrifuge, and take 8 ml. of the supernatant layer. Add 0.2 ml. of a 5% solution of disodium ethylenediamine tetraacetate. After 5 minutes, add 0.05 ml. of fresh 4.8% solution of 1-fluoro-2,4-dinitrobenzene in ethanol. Add 0.4 ml. of a buffer containing 4.2% of sodium bicarbonate and 5.3% of sodium carbonate. Heat at 60° for 20 minutes. Add 1.6 ml. of 0.8% sodium hydroxide solution and continue to heat for an hour. Cool, and add 14 ml. of chlorobenzene. Shake for 3 minutes and centri-

⁵⁷ G. Neurath and E. Doerk, *Chem. Ber.* **97**, 172-8 (1964).

⁵⁸ A. M. Asatoor and D. N. S. Kerr, *Clin. Chim. Acta* **6**, 149-56 (1961).

⁵⁹ J. Bartos, *Talanta* **8**, 619-20 (1961).

fuge. Read the organic phase at 365 $m\mu$ in comparison with a reagent blank. An appropriate standard curve would be that for β -phenylethylamine.

Urine. To 2 ml. of sample diluted with 2 ml. of water, add 1 gram of yellow mercuric oxide and shake for 2 hours. Centrifuge, and remove 2 ml. of the supernatant layer. Add 0.5 ml. of the reagent and 0.4 ml. of the buffer described above for determination in blood. Heat, as there described, before and after addition of sodium hydroxide solution. Dilute to 10 ml. with water and extract with 10 ml. of cyclohexane. Read the organic phase at 350 $m\mu$ against a reagent blank. An appropriate standard curve would be that for piperidine hydrochloride.

By p-nitrophenylazobenzoyl chloride. To about 0.01 mg. of the amine in 2 ml. of dioxan, add 1 ml. of 0.01% solution of *p*-nitrophenylazobenzoyl chloride in dioxan. After 5 minutes, add 2 ml. of 0.05% benzyltrimethylammonium hydroxide in dimethyl sulfoxide and 0.5 ml. of a solution of 0.5 ml. of 30% hydrogen peroxide diluted to 100 ml. with dimethyl sulfoxide. After 10 minutes, read at 510 $m\mu$ against a reagent blank.

TERTIARY AMINES

Tertiary amines react with 2,4,6-trichlorobenzenediazonium fluoroborate.⁶⁰ The resulting methochloride has a strong absorption. Tribenzylamine does not react. Some alkaloids do not follow Beer's law. Primary amines give a small reaction; secondary amines none. The same reagent is applicable to alcohols.

Procedure—Mix 4 ml. of an acetone solution of the amine with 1 ml. of a 10% solution of the reagent in acetone stabilized by 2% of cupric acetate. After 2 hours at room temperature, protected against bright light, read at 400 $m\mu$.

FATTY AMINES

Aqueous solutions of fatty, primary, secondary, and tertiary amines in the range up to 2 ppm. are determined by reaction with methyl orange.⁶¹ In the presence of added salicylaldehyde, the result is for secondary and

⁶⁰ M. Pesez and J. Bartos, *Bull. Soc. Chim. France* **1963**, 2333-4.

⁶¹ Ronald M. Silverstein, *Anal. Chem.* **35**, 154-7 (1963).

tertiary amines; and when acetic anhydride is substituted for the salicylaldehyde, the result is solely the tertiary amine. Acidity is necessarily present to avoid loss of the amine on glass surfaces.

Using primary, secondary, and tertiary octadecylamines, the absorption is at 430 $m\mu$. The color body is believed to be the methyl orange salt of the amine. The color intensity does not change in 24 hours at room temperature. The maximum absorption is at pH 3.4-3.6. Neutral salt is added to facilitate extraction of the color into ethylene dichloride. Excess methyl orange should remain in the aqueous layer. Extraction is about 96% complete. The method is accurate to 0.1 ppm.

Other indicators such as thymol blue, bromocresol green, bromothymol blue, and benzo yellow can be substituted. Chloroform is an alternative solvent. (cf. Amines in feed water, page 67.)

The temperature must be carefully controlled in blocking out the primary amine. With inadequate heating, the reaction is incomplete. Too high a temperature causes some of the secondary amine to react. The reaction with several amines is shown in Table 12. The presence of polyvalent ions does not interfere at concentrations that are low enough so as to not precipitate the amine. The buffer and methyl orange have been combined for field use in determination of total amines.⁶²

An azure blue dye in the presence of copper ion is suitable for development of fatty amines of greater than 10 carbons in a buffer for pH 6.9-7.2.⁶³ As little as 0.1 mg. per liter can be read.

Tertiary amines such as triisooctyl- and trinonylamine are determined as ferric thiocyanate complexes.^{64,65} Secondary amines form these products, but less efficiently. The efficiency is affected by the concentration of ferric and thiocyanate ions and by pH. The same reaction is applicable with cobalt thiocyanates. The method is applicable to Schiff bases in fuel oil.⁶⁶

Procedure—By methyl orange. As a buffer, dissolve 125 grams of potassium chloride and 70 grams of sodium acetate trihydrate in 500 ml. of water. Add 300 ml. of glacial acetic acid and dilute with water to 1 liter. As reagent, dilute 5 ml. of redistilled salicylaldehyde to one liter with ethylene dichloride.

⁶² R. A. Larrick, *ibid.* **35**, 1760 (1959).

⁶³ C. J. Munter, U.S. Patent 2,977,200 (1961); German Patent 1,120,767 (1962).

⁶⁴ M. W. Desai and T. K. S. Murthy, *Analyst* **88**, 322-4 (1963).

⁶⁵ M. W. Desai and T. K. S. Murthy, *Indian J. Chem.* **1**, 106-8 (1963).

⁶⁶ R. E. Heller, *Anal. Chem.* **35**, 1753-4 (1963).

TABLE 12. REACTIONS OF AMINES IN THE METHYL ORANGE PROCEDURE

Compound	Specific absorptivity ($\times 1000$)		
	Test A	Test B	Test C
Ethylamine	<1	0	0
Butylamine ^a	3	3	2
Iso-octylamine ^a	<1	<1	<1
Dodecylamine ^a	260	75	7
Hexadecylamine ^a	351	61	0
Octadecylamine	398	5	0
Behenylamine ^a	299	30	7
Di- <i>n</i> -butylamine ^a	7	7	3
Di-octadecylamine	194	194	0
Triethylamine ^a	10	9	6
Tri- <i>n</i> -butylamine ^a	180	180	114
Tridodecylamine	130	131	132
Tri-octadecylamine	150	148	148
Dodecyldimethylamine ^a	435	434	440
Methyldidodecylamine ^a	284	285	282
Morpholine	0	0	0
Cyclohexylamine	0	0	0
Dibenzylamine ^a	13	12	0
Octadecylamide	0	0	0

^a Unrefined industrial products.

Total amine. (a) Shake 100 ml. of sample with 4 ml. of buffer, 2 ml. of 0.05% methyl orange solution, and 20 ml. of ethylene dichloride for 5 minutes. Allow to separate for 3 minutes and draw off the ethylene dichloride layer. Add 0.5 ml. of isopropanol to solubilize any turbidity and read at 430 $m\mu$ against an ethylene dichloride blank.

Secondary and Tertiary amines. (b) Shake 100 ml. of sample with 4 ml. of buffer and 20 ml. of salicylaldehyde reagent for 2 minutes. Heat at 78° for 15 minutes and cool for 10 minutes. Add 2 ml. of 0.05% methyl orange and shake for 5 minutes. Proceed as for total amine, from "Allow to separate for . . ."

Tertiary amines. (c) Shake 100 ml. of sample with 4 ml. of buffer, 20 ml. of ethylene dichloride, and 1 ml. of acetic anhydride for 10 minutes. Add 2 ml. of 0.05% methyl orange solution and shake for 5 minutes. Complete as for primary amines, from "Allow to separate for . . ."

By azure blue. Dissolve 14 grams of sodium carbonate and 14 grams of sodium bicarbonate in 400 ml. of water. Dissolve 2.58 gram of azure blue, such as Eriochrome azurol B, by shaking. Dilute to 500 ml. with water. Mix 10 ml. of this with 700 ml. of water containing 28 grams of sodium carbonate and 28 grams of sodium bicarbonate, and dilute to 1 liter. Add 1.15 gram of copper sulfate pentahydrate to 15.6 ml. of glacial acetic acid, and dilute to 1 liter with water. Of this, dilute 100 ml. to 1 liter. As acetic acid, dilute 216 ml. of glacial acetic acid to 1 liter with water.

To 500 ml. of sample, add 10 ml. of the diluted acetic acid and 10 ml. of the dilute dye solution. Mix well, add 10 ml. of the diluted copper sulfate solution, and read.

As the ferric thiocyanate complex. To not more than 50 ml. of sample solution containing 0.05-0.5 mg. of test substance, add 10 ml. of cyclohexane. Shake for 2 minutes and discard the aqueous phase. Add 1 ml. of saturated ferric alum solution and 5 ml. of 15.2% ammonium thiocyanate solution. Adjust to about pH 1.5 by addition of 0.5 ml. of 1:9 sulfuric acid and about 5 ml. of water. Shake vigorously and discard the aqueous phase. Centrifuge the cyclohexane to clarify it and read at 490 $m\mu$ against cyclohexane.

As the cobalt thiocyanate complex. Adjust a sample containing 1-5 mg. of amine to pH 1.5 by addition of 1:9 sulfuric acid. Shake for 2 minutes with 10 ml. of carbon tetrachloride. Separate the carbon tetrachloride layer and discard the aqueous layer. Add 3 ml. of 29.1% cobaltous nitrate hexahydrate solution, 10 ml. of 15.2% ammonium thiocyanate solution, and 2 ml. of 1:20 sulfuric acid. Shake for 2 minutes and separate the organic layer. Centrifuge to clarify and read at 620 $m\mu$ against chloroform.

Fuel oil. As a benzene solvent, wash the technical grade successively with 4% sodium hydroxide solution, 1:11 hydrochloric acid, and water. Wash reagent grade isoamyl alcohol with 1:11 hydrochloric acid followed by several water washes. Add 3.5% to the benzene. A reagent blank should not exceed 0.01 in absorbance. As methyl orange reagent, prepare a 0.5% solution in water. Immediately before use, mix with an equal volume of saturated boric acid solution. To prevent loss of amines from aqueous solutions on glass, coat the bottle for shaking with silicone.

Weigh a sample of 4-5 grams expected to contain about 0.01% of Schiff

bases, tertiary dodecyl to pentadecyl derivatives of $-\text{N}:\text{CH}_2$. Dissolve in benzene containing 1.5% of isoamyl alcohol. Dilute to 50 ml. Wash with 10 ml. of 10% sodium hydroxide solution, with 2 minutes of shaking. Similarly wash with 10 ml. of water.

To 25 ml. of the benzene solution in a 100-ml. silicone-treated glass-stoppered bottle, add 1 ml. of the reagent. Shake vigorously for 5 minutes. Decant about 15 ml. of the supernatant layer and centrifuge for 3 minutes at 1500-2000 rpm. Add 10 ml. of this to 20 ml. of 1:11 hydrochloric acid and shake for 1 minute. Isolate the aqueous phase, centrifuging if necessary. Read at 508 and 700 $m\mu$ against water. As reference curve, use the difference between the two readings.

n-BUTYLAMINE

n-Butylamine may be determined in air by reaction with ninhydrin.⁶⁷ For a discussion of the ninhydrin determination of amines, see page 74. Beer's law is followed for 0.002-0.1 mg. per ml.

n-Butylamine reacts with 1,2-naphthoquinone-4-sulfonate at pH 10.3 to give a red color. This is extracted with chloroform and read at 450 $m\mu$.⁶⁸ The procedure is applicable for concentrations up to 2 ppm.

Procedure—Air by ninhydrin. Draw a measured volume of air through a measured volume of a 1:99 mixture of concentrated hydrochloric acid and isopropanol. To a 3-ml. portion of the absorbing solution, add 5 ml. of pyridine and 2 ml. of 0.2% ninhydrin solution in isopropanol. Dilute to 10 ml. with the acid-isopropanol mixture. Stopper, mix, and heat at 85° for 7 minutes. Immerse in cold running water for 10 minutes. Read at 575 $m\mu$. To calculate the ppm. of butylamine in air:

$$\text{ppm.} = (P \times F \times 25 \times 10^6) / (73.14 \times V \times M \times 28320)$$

in which

- ppm. = cubic centimeters of *n*-butylamine vapor per cubic meter of air
 P = total mg. *n*-butylamine in 10 ml. of colored solution
 F = $\frac{\text{total ml. sampling solution}}{\text{ml. sampling solution used as aliquot}}$
 25 = cubic centimeters of vapor in 1 millimole of any substance at room temperature

⁶⁷ Richard F. Scherberger, Franklin A. Miller and David W. Fassett, *Am. Ind. Hyg. Assoc. J.* **6**, 471-4 (1960).

⁶⁸ David H. Rosenblatt, Peter Hlinka and Joseph Epstein, *Anal. Chem.* **27**, 1290-3 (1955).

73.14 = millimolecular weight of butylamine in milligrams
 V = sampling rate in cubic feet per minute
 M = length of air sampling period in minutes
28320 = factor used to convert cubic feet to cubic centimeters

By *1,2-naphthoquinone-4-sulfonate*. Follow the procedure for ethyleneimine with this reagent, extending the reaction to 20 minutes and reading at 450 $m\mu$.

TRIS, *Tris* (HYDROXYMETHYL) METHYLAMINE

For determination, *tris* is oxidized in acid solution and read at 400 $m\mu$.⁶⁹ A preferred concentration is 20-90 mg. of *tris* per 100 ml.

Procedure—*Plasma*. Add 0.5 ml. of deproteinized sample to a 0.2-gram column of Dowex 50-X8 in the hydrogen form. Follow it with 0.5 ml. of water. After 5 minutes, wash the column with water. Elute the *tris* with 1 ml. of 1:4 sulfuric acid. To 1 ml. of eluate, add 0.3 ml. of 0.2% potassium dichromate solution in 1:1 sulfuric acid. Heat at 100° for 30 minutes and cool. Read at 400 $m\mu$.

HEXAMINE, HEXAMETHYLENETETRAMINE, METHENAMINE

Hexamine in waste waters is converted to formaldehyde and determined by phenylhydrazine hydrochloride⁷⁰ (cf. Vol. IV, p. 56). Free formaldehyde is separately determined.

Procedure—To a 50-ml. sample, add 5 ml. of concentrated sulfuric acid. Distil until white fumes appear. Dilute the distillate to 100 ml. Mix 8 ml. of the distillate with 2 ml. of filtered 5% phenylhydrazine hydrochloride solution. After 10 minutes, add 10 ml. of 30% sodium hydroxide solution and 10 ml. of isopropanol. Read at 530 $m\mu$. To calculate, subtract a determination of free formaldehyde in the original solution.

ETHYL, DIETHYL, AND TRIETHYLAMINE

The reaction of the amines with hypochlorite and development with starch-potassium iodide is a complex function of pH.⁷¹ This method was

⁶⁹ J. Strauss, K. Bernath and S. A. Kaplan, *Proc. Soc. Exptl. Biol. Med.* **113**, 58-61 (1963).

⁷⁰ V. Stankovič, *Chem. Zvesti* **17**, 274-9 (1963).

⁷¹ George Dahlgren, *Anal. Chem.* **36**, 596-9 (1964).

developed originally for ammonia.⁷² Properly applied, the reaction is sensitive in the parts per billion range. The first step of reaction with hypochlorite forms the chloro derivative. Excess hypochlorite is destroyed by nitrite without affecting the chloramine. The subsequent reaction with starch-potassium iodide is extraordinarily sensitive. At a pH of 5.2, amines, amides, and ammonia react. At pH 6.6, amines and some amides react, but low concentrations of ammonia do not. At pH 8.1, amines react but ammonia and most amides do not. Addition of bromide ion at pH 8.1 renders amides undetectable and increases the sensitivity for ethylamine. Then the sensitivity for diethylamine is not affected, but that for triethylamine disappears.

The starch-potassium iodide solution, if protected from light at 10°, remains colorless for weeks and does not mold. Linear starch should be used. Ordinary soluble starch may give values as much as 40% lower. Ordinary distilled water gives a light-blue blank at pH 5.2 due to residual ammonia. Colorless blanks are obtained at pH 6.6 and 8.1. In some cases, at pH 8.1, fading begins after 5 minutes.

At this pH, acetamide and pyridine do not interfere, ammonia interferes above 1.2%, and maleamic acids, aniline, and ethyl anilines do interfere. At pH 8.1, there is no interference by ammonia, acetamide, *N*-substituted maleamic acids, and *N*-substituted anilines. There is slight interference by aniline and maleamic acid. Increase of temperature decreases the absorbance.

Excess bromide ion decreases the absorbance at pH 5.2 due to destruction of bromamines by nitrate. This is rapid with ethylamine, slower with diethylamine. At pH 6.6, there is a slight decrease in absorbance by ethyl- and diethylamine.

At pH 8.1, excess bromide ion multiplies the molar absorptivity of ethylamine by 1.5. The bromide ion does not affect the result with diethylamine. Bromide ion prevents reaction of triethylamine due to reduction of the bromamine by nitrite ion. Acetamide, aniline, and *N*-substituted maleamic acids interfere at pH 8.1 when bromide ion is present. Maleamic acid, pyridine, and *N*-substituted anilines do not interfere.

Procedure—As a buffer for pH 5.2, dissolve 136 grams of sodium acetate trihydrate and 21.2 ml. of glacial acetic acid in water and dilute to 1 liter. As a buffer for pH 6.6, dissolve 25 grams of potassium dihydrogen phosphate per 100 ml. of solution. Add 50 per cent sodium hydroxide to adjust the pH to 6.05. Addition of 1 ml. of this to a sample solution gives

⁷² F. Zitomer and J. L. Lambert, *ibid.* **34**, 1738-40 (1962).

the pH of 6.6. As the developer of color, dissolve 7.5 grams of linear soluble starch in 1 liter of boiling water. After 15 minutes, slowly add 10 grams of potassium iodide. Boil for 5 minutes and cool. As hypochlorite, use 2.5 ml. of Zonite per 100 ml. of solution.

pH 5.2. To 25 ml. of amine sample, add 1 ml. of buffer for pH 5.2 and 1 ml. of stock hypochlorite solution. After 4 minutes for ethylamine or 10 minutes for triethylamine, add 1 ml. of 0.5% sodium nitrite solution. One minute later, add 1 ml. of starch-potassium iodide solution. After 15 minutes, read at 540 $m\mu$. This pH is not applicable for triethylamine.

pH 6.6. Follow the technic for pH 5.2 but with the buffer for pH 6.6.

pH 8.1. To a 25-ml. sample of amine solution, add 0.5-1 gram of sodium bicarbonate. Add 1 ml. of hypochlorite reagent. After 1 minute for ethylamine or diethylamine, or 1 minute per micromole of triethylamine per liter, add 1 ml. of 0.5% sodium nitrite solution. After another minute, add 1 ml. of starch-potassium iodide reagent. After 3 minutes, read at 540 $m\mu$.

Calibration curves obtained with hypochlorite and iodine solutions are satisfactory. Molar absorptivities are shown in Table 13. Those for hypo-

TABLE 13. MOLAR ABSORPTIVITIES OF ETHYLAMINES AT 540 $m\mu$. $T = 25 \pm 2^\circ\text{C}$.

<i>Compound</i>	<i>Procedure pH</i>	<i>Molar absorptivity</i>
Ethylamine	5.2	31,000
	6.6	30,800
	6.6 (Br^-)	30,900
	8.1	26,400
	8.1 (Br^-)	44,600
Diethylamine	5.2	20,800
	6.6	22,900
	6.6 (Br^-)	20,500
	8.1	22,200
	8.1 (Br^-)	22,500
Triethylamine	8.1	14,000
Ammonia	5.2	30,500
Hypochlorite	2.7	20,500 (570 $m\mu$)
Iodine	3.2	19,600 (570 $m\mu$)

chlorite below pH 4 and iodine above pH 4.0 are the same at 570 and 540 m μ .

FORMAMIDE

Ammonium formate, formic acid, water and ammonium ion do not interfere with determination of formamide by ferric alum.⁷³ Conditions for determination of formamide as its hydroxamic acid are shown in Volume IIIA, pages 314-6.

Procedure—To prepare the reagent, dissolve 40 grams of ferric alum and 100 ml. of 1:3 hydrochloric acid in water and dilute to 200 ml. with water. Mix 25 ml. of aqueous solution containing up to 3 mg. of formamide per ml. with 5 ml. of 10% sodium hydroxide solution and 5 ml. of 7% hydroxylamine hydrochloride solution. Heat at 40° for 40 minutes. Cool in ice water for 3 minutes and let stand until the solution reaches room temperature. Add 30 ml. of water, 5 ml. of 1:4 nitric acid, and 10 ml. of the ferric alum reagent. Read at 490 m μ at 10 minutes after addition of the ferric alum solution.

DIMETHYLFORMAMIDE

Dimethylformamide in hydrocarbons is determined by a method not unlike that shown for formamide.⁷⁴ However, incomplete extraction is to hydrolyze to dimethylamine and develop with furfural. Accuracy is of the same order of magnitude.

Dimethylamine is also determined as the copper complex.⁷⁵ After hydrolysis, the dimethylformamide is similarly determined by difference.

Procedure—*By ferric alum.* Mix 2 ml. of 14% sodium hydroxide solution with a hydrocarbon sample containing 0.3-1 mg. of dimethylformamide and 2 ml. of 21% hydroxylamine hydrochloric solution. Heat at 40° for 40 minutes. Cool and add 2 ml. of 1:4 nitric acid and 2 ml. of

⁷³ Satoshi Mizukami and Koichi Nagato, *Ann. Rept. Shionogi Research Lab.* **6**, 64-7 (1956).

⁷⁴ L. S. Kofman, V. S. Vinogradova and V. B. Mitrofanova, *Vestn. Tekhn. i Ekon. Inform., Nauch.-Issled. Inst. Tekhn.-Ekon. Issled. Gos. Kom. Sov. Min. SSSR* **20**, *Khim.* **1961**, No. 11, 21-6.

⁷⁵ V. S. Fikhtengol'ts, R. V. Zolaterava, I. Ya. Poddubnyi and A. V. Khoroshin, *Zavod. Lab.* **29**, 160-1 (1963).

saturated ferric alum solution. Mix well, let stand, and read the aqueous layer.

As dimethylamine. Heat 5-100 ml. of the hydrocarbon with 5 ml. of 10% sulfuric acid at 60° for 1 hour. Cool, pour off the acid layer, and wash the hydrocarbon layer with water. Make the combined acid layer and washings alkaline with 20% potassium hydroxide solution. Distil the dimethylamine into 2 ml. of 1:25 hydrochloric acid. Add 2.5 ml. of 0.56% potassium hydroxide solution and 1.5 ml. of 25% furfural solution. Read after 30 minutes.

By cupric ion. Isoprene. As reagent, mix 50 grams of sodium hydroxide in 100 ml. of water with 100 ml. of concentrated ammonium hydroxide and 100 grams of ammonium acetate and 1 gram of copper sulfate pentahydrate in 150 ml. of water. Dilute to 500 ml.

Extract a 75-ml. volume as measured at 0° with 25 and 25 ml. of 1:220 hydrochloric acid followed by 25 ml. of water. Dilute to 100 ml. and use 25 ml. as sample. Add 2 ml. of copper reagent and 20 ml. of 5% carbon bisulfide in benzene. Place the closed flask in a 45° bath for 5 minutes. Shake vigorously for 1 minute. Add 2 ml. of 30% acetic acid and again shake for 1 minute. Separate the benzene layer and read with a blue filter for dimethylamine.

To 40 ml. of the benzene layer, add 20 ml. of 30% sodium hydroxide solution. Heat at 100° and pass air at 200 ml. per minute, absorbing the resulting dimethylamine in 15 ml. of 1:1000 hydrochloric acid. Determine dimethylamine as before. The difference properly translated is dimethylformamide.

OXAMIDE

Oxamide, the amide of oxalic acid, is hydrolyzed with 10% sodium hydroxide, steam distilled, and determined by the liberated ammonia using the Nessler reaction.⁷⁶ The method was developed for its determination in nitrocellulose-base propellants. A preliminary extraction with methylene chloride removes nitroglycerin, dinitrotoluene, diphenylamine, and ethyl centralite. The first two of these would cause erratic results. The oxamide must be hydrolyzed in the presence of the nitrocellulose. No method of separation is known. Each gram of oxamide yields 0.387

⁷⁶ Julius B. Apatoff, Joseph Cohen and George Norwitz, *Anal. Chem.* **35**, 800-2 (1963).

gram of ammonia. This is yielded so slowly in the steam distillation that it is not necessary to use acid in the receiver. A blank corrects for ammonia from hydrolysis of the nitrocellulose which approximates 1.7 mg. Ammonium chloride may replace oxamide in preparation of a calibration curve.

Procedure—Extract a 1-gram sample overnight in a Soxhlet apparatus with methylene chloride. Disassemble and let the solvent evaporate from the thimble. Assemble a steam distillation apparatus with a 2-liter flask for water, a 1-liter flask for the sample, and a 750 ml. flask as receiver. Add about 1500 ml. of water to the appropriate flask. Put the sample in the 1-liter flask and add 100 ml. of water to the receiver. Add 150 ml. of 10% sodium hydroxide solution to the sample. Heat the water flask but not the sample flask and distil at 5-7 ml. per minute until 225-250 ml. has been collected. Run a blank with nitrocellulose of 12.2-13.3% nitrogen. Dilute the distillate to 500 ml.

As Nessler reagent, dissolve 70 grams of potassium iodide in 60 ml. of water. Add 100 grams of mercuric iodide and swirl. Dilute to about 150 ml. and slowly add with stirring to a cold solution of 160 grams of sodium hydroxide in 500 ml. of water. Dilute to 1 liter and let stand at least overnight. Dilute a 5-ml. aliquot of the distillate to about 45 ml. Add 1 ml. of Nessler reagent and mix. Dilute to 50 ml. and mix. After 15-30 minutes, read at 415 m μ with a reagent blank as zero.

AMINOACETONE

For the determination of aminoacetone by picric acid, see Δ -amino-levulinic acid, page 129.

2-DIETHYLAMINOETHANOL

2-Diethylaminoethanol is determined by cobaltous chloride and *o*-dianisidine.⁷⁷ See 1,2-*bis*(methythio)ethane, Volume IIIA, page 454 for discussion.

Procedure—As a reagent, dissolve 0.2 gram of cobaltous chloride hexahydrate in 100 ml. of water and add 2 drops of 30% hydrogen perox-

⁷⁷ Jacob S. Harker, Irwin Master, Louis E. Mattison and Benjamin Witten, *Anal. Chem.* **29**, 82-4 (1957).

ide. To a 1-ml. sample, add 1 ml. of reagent, 0.2 ml. of 0.04% *o*-dianisidine solution in absolute ethanol, and 2 ml. of a buffer for pH 4. Read within 30 seconds at 454 $m\mu$.

FATTY ACID AMIDES

By reaction with alkaline hydroxylamine, fatty acid amides form hydroxamic acids, which give a purple iron chelate complex with ferric perchlorate.⁷⁸

Procedure⁷⁹—To prepare the ferric perchlorate reagent, dissolve 5 grams in 10 ml. of 70% perchloric acid and 10 ml. of water. Dilute to 100 ml. with cold absolute ethanol. Dilute a 4-ml. aliquot of this solution and 3 ml. of 70% perchloric acid to 100 ml. with cold absolute ethanol. Dissolve 4 grams of sodium hydroxide in 2.5 ml. of water. Dilute to 50 ml. with absolute ethanol. Separate sodium chloride by centrifuging. Dissolve 2 grams of hydroxylamine in 2.5 ml. of water and dilute to 50 ml. with absolute ethanol. Mix equal volumes of ethanolic hydroxylamine and sodium hydroxide solutions.

To a 5-10-mg. dry lyophilized sample containing 1-5 milliequivalents of fatty acid as ester or amide, add 2 ml. of alkaline hydroxylamine reagent. Seal and heat at 67° for 5 hours. Cool to room temperature and add 5 ml. of ferric perchlorate reagent. After 30 minutes, read the purple color at 530 $m\mu$. If necessary, centrifuge to remove insoluble polysaccharides before reading.

PHENYLACETAMIDE

Phenylacetamide and its neutral derivatives, *N*-methyl- or *N*-(2-hydroxyethyl)-phenylacetamide, are determined as phenylacetic acid after hydrolysis.⁸⁰ The latter is nitrated and then reduced with hydroxylamine in ammoniacal solution to give a stable violet color. For discussion of the reaction, see Volume IIIA, pages 322-3. The phenylacetamides are first extracted from interferences with chloroform from an alkaline solution. The chloroform is evaporated at a low temperature to avoid sublimation.

⁷⁸ Willard T. Haskins, *Anal. Chem.* **33**, 1445-6 (1961).

⁷⁹ Fred Snyder and Nelson Stephens, *Biochim. et Biophys. Acta* **34**, 244-5 (1959)

⁸⁰ S. C. Pan and David Perlman, *Anal. Chem.* **26**, 1432-38 (1954).

Procedure—*Penicillin fermentation medium*. Add 0.1 ml. of 4% sodium hydroxide solution to a 1-ml. sample, so that it becomes blue to thymol blue. Add 0.3 gram of anhydrous sodium sulfate and shake for 30 seconds with 10 ml. of chloroform. Cork loosely and centrifuge for 5 minutes. Remove the upper aqueous layer with suction and evaporate 8 ml. of the chloroform layer to dryness at 30-35° under a gentle current of air.

Dissolve the residue in 1 ml. of 20% sodium hydroxide solution and heat at 100° for 1 hour with the tube loosely corked. Carefully neutralize the hydrolyzate by dropwise addition of 0.2 ml. of concentrated sulfuric acid, keeping the tube in cold water. The resulting solution should be acid to thymol blue. Alkalize an aliquot containing up to 0.5 mg. of phenylacetic acid with 0.1 ml. of 4% sodium hydroxide solution. Dry at 50-70° under a gentle current of air. Add 0.25 ml. of a nitrating agent prepared by mixing 20 grams of potassium nitrate, 100 ml. of concentrated sulfuric acid, and 10 ml. of water. Heat at 40-44° for 15 minutes and add 0.5 ml. of 15% hydroxylamine hydrochloride solution. Mix and add 6.25 ml. of 1:3 ammonium hydroxide. Mix, and let stand at room temperature for 1 hour. Read at 540 m μ against a reagent blank within 30 minutes.

POLYACRYLAMIDES

Polyacrylamides in water are determined photometrically by Nessler's reagent.⁵¹ Alternatively, they are determinable nephelometrically by an appropriate cationic agent.

Procedure—*Photometric*. Concentrate 1 liter of water to under 10 ml. and cool. Add 20 ml. of 1:1 sulfuric acid. Boil for 30 minutes just below fuming and cool. Add 400 ml. of water and 16 grams of sodium hydroxide. Distil 250 ml. Take an aliquot of the distillate, add 5 ml. of Nessler's reagent, page 332, and dilute to a known volume. Mix, and after 30 minutes, read at 425 m μ .

Nephelometric. Pass the sample through an ion-exchange column, then evaporate to under 10 ml. Dilute to 10 ml. and add 1 ml. of 10% sodium hydroxide solution. Heat at 100° and cool. Add 1 drop of 1% phenolphthalein indicator solution in 95% ethanol and titrate accurately with 1:110 hydrochloric acid to disappearance of the color. Mix with 1 ml.

⁵¹ W. B. Crummett and R. A. Hummel, *J. Am. Water Works Assoc.* **55**, 209-19 (1963).

of 3% sodium citrate dihydrate and dilute to 50 ml. Add 0.1 ml. of 4% diisobutylphenoxyethoxyethyltrimethylbenzylammonium chloride (Hyamine 1622). Read the turbidity nephelometrically.

α -AMINONITRILES

α -Aminonitriles are developed by reaction with aqueous bromine solution followed by benzidine-pyridine reagent. The method is suitable for determining aminoacetonitrile, α -aminopropionitrile, α -aminobutyronitrile, phenylaminoacetonitrile, methyleneaminoacetonitrile, and imino-dioacetonitrile in the range of 0.01-0.1 micromoles.⁸² β -Alanine, DL- α -alanine, L-glutamic acid and L-glutamine in concentrations of 10^{-7} M do not interfere in the determination of aminoacetonitrile, α -aminopropionitrile and methyleneaminoacetonitrile. Cysteine and glutathione in concentrations exceeding 10^{-8} M interfere.

Procedure—To prepare the 1:3 benzidine-pyridine mixture, dissolve 1 gram of benzidine in 13 ml. of hot 1:10 hydrochloric acid and dilute to 30 ml. with water. Add 20 ml. of concentrated hydrochloric acid to 80 ml. of water. Add 120 ml. of pyridine. Mix 1 part of the benzidine solution with 3 parts of the pyridine solution.

Mix 0.5 ml. of an aqueous solution containing about 0.0001 M α -aminonitrile with 5 ml. of 10% trichloroacetic acid. Add 0.25 ml. of a saturated aqueous bromine solution. Cover and heat at 37° for 1 hour. Cool, add 0.25 ml. of sodium arsenite prepared by dissolving 2 grams of arsenic trioxide in 100 ml. of hot 0.4% sodium hydroxide solution. Add 3.5 ml. of the benzidine-pyridine reagent and let stand for 15 minutes. Read at 530 m μ against a standard developed with 0.00065% potassium cyanide solution.

β -AMINOPROPIONITRILE

β -Aminopropionitrile forms a green color when treated with ninhydrin, in contrast to the red color usually formed with amines.⁸³ The resulting β -(N- γ -L-glutamyl)aminopropionitrile is extracted from the sample and hydrolyzed. The resulting free β -aminopropionitrile is separated from interfering ninhydrin reacting substances by liquid-liquid extraction using ether. Hydrazine and phthalhydrazine give green shades with the

⁸² Apolinary Szewczuk, *Chem. Anal.* **4**, 971-9 (1959).

⁸³ J. T. Garbutt and G. M. Strong, *J. Agr. Food Chem.* **5**, 367-70 (1957).

reagent. As little as 50 ppm. of β -aminopropionitrile may be developed and the color is stable for 30 minutes.

Procedure—*Legume seeds*. Mix a 60-80 mesh sample, containing at least 0.1 mg. of β -aminopropionitrile, with 20-30 ml. of water. Add a drop of antifoaming agent and several porcelain boiling chips. Reflux for about 40 hours. Filter, and wash the residue with three 3-ml portions of water. Dilute the combined filtrate and washings to 25-35 ml. Using a liquid-liquid extractor, add a 15-25 ml. aliquot to the sample tube so that the solution fills a depth of 8-10 cm. Saturate with solid potassium chloride and adjust the pH to 10 with anhydrous potassium carbonate. Extract with ether at a reflux rate of 2-4 drops per second for 24 hours. Evaporate the ether extract at 100° and take up the residue with 1-butanol. Dilute with 1-butanol to 25 ml.

To prepare the sodium hydroxide solution, dissolve 0.5 gram in 20 ml. of 1-butanol, let stand for 4-5 hours, and filter. Prepare duplicate 0.2-, 0.4-, 0.6-, 0.8-, and 1-ml. aliquots of β -aminopropionitrile standard solution in 1-butanol containing 0.1 mg. per ml. Add 1 ml. of 2% ninhydrin solution in 1-butanol and dilute each tube to 12 ml. with 1-butanol. Prepare duplicate 0.5-, 1.0-, 2.0-, and 5.0-ml. aliquots of the sample solution and add 1 ml. of 2% ninhydrin solution in butanol to each. Dilute each to 12 ml. with 1-butanol. Stopper with a rubber stopper containing an 8-cm length of 6-mm. glass tubing. Heat at 100° for 20 minutes. Cool for 2 minutes in 10-20° water and add 0.05 ml. of sodium hydroxide solution to each. Heat at 100° for 4 minutes and cool for 2 minutes in 10-20° water. Read each standard tube at 640 $m\mu$ against a reagent blank and calculate the samples by interpolation of the standard curve. Using only those values that fall on the steeply rising part of the curve, from about 0.02-0.08 mg. of β -aminopropionitrile per tube, calculate the amine per ml. of sample for each tube. Average the content for those samples on the rising part of the curve.

METHYL *bis*(2-CHLOROETHYL)AMINE *N*-OXIDE

Beer's law is followed for 0.01-0.2 mg. per ml. at 410 $m\mu$ with bromocresol purple as the reagent.⁸⁴

Procedure—*Biological fluids*. To a 5 ml. sample containing 0.1-2 mg. of methyl *bis*(2-chloroethyl)amine *N*-oxide, add 10 ml. of hexane, 1 ml. of 1:10 hydrochloric acid, and 1 ml. of 5.2% sodium bisulfite solution.

⁸⁴ Mataichiro Minami, *Osaka Shiritsu Daigaku Igaku Zasshi* 9, 1483-1508 (1960).

After 5 minutes, add 1 ml. of 17% potassium hydroxide solution and centrifuge. Separate 8 ml. of the upper hexane layer, add 8 ml. of 1:110 hydrochloric acid, and centrifuge. Separate 7 ml. of the hydrochloric acid layer. Add an equal volume of hexane and 1 ml. of 12% potassium hydroxide solution. Centrifuge to separate the hexane layer. To the hexane layer, add 0.5 ml. of 0.05% bromocresol purple solution in 95% ethanol. Mix, and after 1 minute, read at 410 m μ .

2-AMINO-2-HYDROXYMETHYL-1,3-PROPANEDIOL

When amines are oxidized with potassium dichromate, the yellow of the dichromate disappears and the reduced green chromic ion appears. The disappearance of the dichromate is measured as an indirect method for determination of amines rather than the appearance of the reduced chromic ion, since the green has little absorption in the useful range of the spectrophotometer.⁸⁵ With 0.2% potassium dichromate solution, 0.2-10 mg. of 2-amino-2-hydroxymethyl-1,3-propanediol is measured. By diluting the reagent to 0.04%, 0.02-0.2 mg. per ml. may be determined. Interference from serine, threonine, choline, and ethanolamine is removed by passage through Dowex 50.

Procedure—To prepare the exchange agent, vigorously stir 60-100 mesh Dowex 50, H⁺ form, with water and allow to settle. Decant the fine floating material.

Shake a sample diluted to 5 ml., containing 0.2-10 mg. of 2-amino-2-hydroxymethyl-1,3-propanediol per ml., with 1 gram of the Dowex for 3 minutes. Decant the sample. Wash the Dowex several times with water. Add 10 ml. of water, decant, and add 5 ml. of 1:5 sulfuric acid. Swirl for 2 minutes and dilute to 50 ml. with water. Add exactly 3 ml. of 0.2% potassium dichromate solution in 1:1 sulfuric acid. Heat at 100° for 30 minutes and read at 450 m μ .

For samples containing 0.02-0.2 mg. per ml., add 3 ml. of 0.04% potassium dichromate solution in 1:1 sulfuric acid and read at 255 m μ .

ETHYLENIMINES, VINYL AMINES

γ -(4-Nitrobenzyl)pyridine is a reagent for *ethylenimine*, *2-ethylethylenimine*, *2,2-dimethylethylenimine* and *N-ethylethylenimine*.⁸⁶ The determination is carried out in water. For estimation of reactive alkylating

⁸⁵ Hyman Rosen, *Ann. N.Y. Acad. Sci.* **92**, 414-18 (1961).

⁸⁶ Joseph Epstein, Robert W. Rosenthal and Richard J. Ess, *Anal. Chem.* **27**, 1435-9 (1955).

materials in the presence of nonreactive alkylating materials by this reagent, the medium should be non-aqueous. Ethylamine and ethanolamine, the hydrolysis product of ethylenimine in concentrations as much as 1000 times that of ethylenimine do not interfere. Beer's law is followed for 0.5 ppm. of imine.

Procedure—To a 3-ml. sample solution containing up to 5 ppm. of ethylenimine, 2-ethylethylenimine, 2,2-dimethylethylenimine and *N*-ethylethylenimine, add 1 ml. of a buffer at pH 4, and 1 ml. of 5% γ -(4-nitrobenzyl) pyridine solution in methyl ethyl ketone or acetone. Mix, heat at 100° for 20 minutes, and cool in ice water. Add 4 ml. of acetone and 1 ml. of 14% potassium carbonate solution, and dilute to 10 ml. with water. Mix, and read immediately at 600 $m\mu$ against water. Subtract the absorbance of a reagent blank from that of the sample.

ETHYLENIMINE, VINYL AMINE

Ethylenimine reacts with 1,2-naphthoquinone-4-sulfonate at pH 10.3 to give a red color.⁸⁷ Extraction with chloroform eliminates color from unreacted reagent. Ethanolamide, a product of the hydrolysis of ethylenimine, also forms a red color with the reagent. This is not extracted with chloroform and therefore does not interfere. Differential extraction is accomplished with isoamyl alcohol to read the ethanolamide at 420 $m\mu$. *n*-Butylamine also reacts with the reagent to give a red color, which is read at 450 $m\mu$. Beer's law is followed up to 0.5 ppm. of ethylenimine at 420 $m\mu$.

Procedure—Mix a 50-ml. aqueous ethylenimine solution containing up to 0.5 ppm. of ethylenimine with 10 ml. of 0.138% potassium 1,2-naphthoquinone-4-sulfonate solution and 1 ml. of 0.05 *M* phosphate buffer at pH 11.7 (Vol. 1, p. 175). After 1 minute, add 10 ml. of chloroform and a teflon-covered magnetic stirring bar. Stopper, and stir vigorously for 5 minutes. Allow the layers to separate and pipet off the chloroform layer. Read at 420 $m\mu$ against a chloroform blank.

JADIT, 4-CHLORO-2-HYDROXYBENZOIC ACID BUTYLAMIDE

Jadit is read by fluorescence in serum samples. The reading is at pH 3 to prevent formation of decomposition products.⁸⁸

⁸⁷ David H. Rosenblatt, Peter Hlinka and Joseph Epstein, *Anal. Chem.* 27, 1290-3 (1955).

⁸⁸ A. Häussler and P. Hajdu, *Arzneimittel-Forsch.* 13, 16-17 (1963).

Procedure—*Scrum*. Shake a 1-ml. sample with 5 ml. of butanol, and centrifuge. Mix 3 ml. of the butanol layer with 2 ml. of 1:20 hydrochloric acid and centrifuge. Excite at 400 m μ and read the fluorescence at 305 m μ .

ETHYLENEDIAMINE

The cobalt salt of ethylenediamine reduces a tetrazolium salt to a colored formazan.⁸⁹ The cobalt salt of ethylenediamine tetraacetic acid does not so react. The color conforms to Beer's law up to 100 mg. per liter. There is no reaction with ammonia, pyridine, aniline, 2-aminodimethylamine, or 2-aminodiethylamine.

Procedure—As reagent, prepare a saturated solution of 3-*p*-iodophenyl-2-*p*-nitrophenyltetrazolium chloride in hot water and filter. Store at 0° in an amber bottle. Prepare a 5% aqueous gelatine solution. Store at 0° in an amber bottle, but warm to liquify for use. As the color reagent, mix two parts of the tetrazolium solution with 1 part of the gelatine solution. As a buffer, dissolve 121.13 gram of *tris*(hydroxymethyl)amino-methane in 800 ml. of water. Adjust the pH to 10.5 with 1:1 hydrochloric acid and dilute to 1 liter.

To 1 ml. of aqueous sample solution, add 1 ml. of 0.1% cobaltous chloride solution. Add 1.5 ml. of the color reagent and 1 ml. of the pH 10.5 buffer. Shake at 60° for 30 minutes. Cool, and read at 500 m μ .

EDTA, ETHYLENEDIAMINE TETRAACETATE, (ETHYLENEDINITRILO) TETRAACETIC ACETATE

Bismuth forms a red-violet 1:1 complex with Pyrocatechol Violet at pH 2.1-2.6 which can be read at 580 m μ .^{90,91,92,93} This is decolorized at pH 2.2 by ethylenediamine tetraacetate (EDTA).⁹⁴ The reaction conforms to Beer's law over the range of 0.7-10.4 microgram per ml. of final solution.

The molar ratio of aluminum must not be more than 1.8 times the EDTA. If iron is reduced by ascorbic acid, it may be present at 4.5 times

⁸⁹ J. S. Harker, M. D. Sulkin, Michael Gilman and A. M. Seligman, *Anal. Chim. Acta* **28**, 150-5 (1963).

⁹⁰ V. Suk and Miroslav Malát, *Chemist Analyst* **45**, 30-7 (1956).

⁹¹ Miroslav Malát, *Naturwissenschaften* **48**, 569 (1961).

⁹² Miroslav Malát, *Z. anal. Chem.* **186**, 418-23 (1962).

⁹³ A. Štáhlavská and Miroslav Malát, *Českosl. Farm.* **13**, 89-93 (1964).

⁹⁴ Miroslav Malát, *Chemist Analyst* **51**, 74-5 (1962).

the moles of EDTA. Fluoride, iodide, oxalate, or citrate must not be more than 2×10^{-6} molar. Alkali metals, ammonium ion, and rare earths do not interfere. The following metals interfere by forming complexes with the dye at pH 2.2: nickel, stannic, zirconium, titanium, indium, gallium, hexavalent molybdenum and tetravalent tungsten.

This sequestrant can be determined in the presence of calcium and magnesium ions by its reduction in the color of ferric thiocyanate.⁹⁵ The color decreases linearly. The absorbance has a constant value at pH 2.5-3.5. The lack of interference by calcium or magnesium ions is due to the high stability constant of the iron-EDTA complex. Orthophosphate and adenosine-5'-phosphate interfere at over 0.5 micromole. Adenosine di- and triphosphate interfere above 0.05 micromolar.⁹⁶

Alternatively, after addition of excess ferric ion, the unsequestered iron is read by salicylic acid.⁹⁷ Carrying this principle further, ferrous 2,4,6-tripyridyl-s-triazine has been used for microquantities.⁹⁸

EDTA is determined by its effect in decolorizing ferric sulfosalicylate.⁹⁹ The identical reaction is given by diethylenetriaminepenta-acetic acid and by diaminobutanetetra-acetic acid.

Hydrated chromic ion reacts too slowly with EDTA to be satisfactory. This is resolved by creating unhydrated chromic ion in the presence of EDTA by reduction of dichromate.¹⁰⁰ Zinc ion in large excess serves to prevent formation of the chromium complex in a correction blank.

The cobalt chelate (Volume IIIA, pp. 370-1) gives a violet complex, which develops slowly and, at its maximum, is sensitive to 2 ppm.¹⁰¹

Nickel will displace calcium, magnesium, and many other metals from their EDTA complexes. The unsequestered nickel is precipitated with dimethylglyoxime. The sequestered nickel is then liberated in strongly acid solution and read as the compound with potassium di-thiooxalate.¹⁰² The method will detect less than 0.5 mg. of EDTA per

⁹⁵ Charles J. Parker, Jr., *Anal. Chem.* **38**, 236-7 (1964).

⁹⁶ Oscar Menis, H. P. House and I. B. Rubin, *Anal. Chem.* **28**, 1439-41 (1956).

⁹⁷ G. W. F. Brady and J. R. Gwilt, *J. Appl. Chem.* (London) **12**, 79-82 (1962).

⁹⁸ Byron Kratochvil and Martha Clasby White, *Anal. Chem.* **37**, 111-13 (1965).

⁹⁹ A. Hladka, V. Zbořil, and A. Peškova, *Pracovní Lék* **16**, 447-51 (1964).

¹⁰⁰ P. J. Cherney, Barbara Crafts, H. H. Hagermoier, A. J. Boule, Ray Harbin and Bennie Zak, *Anal. Chem.* **26**, 1806-9 (1954); Robert E. Mosher, Patricia J. Burear and A. J. Boule, *ibid.* **35**, 403-4 (1963).

¹⁰¹ G. Seris, *Ann. fals et fraudes* **47**, 29-30 (1954); J. Vogel and J. Deshusses, *Mitt. Ghibite Lebensm. Hhg.* **53**, 175-8 (1962); S. Wakimoto and T. Akiyama, *Bansetsu Kagaku* **10**, 971-4 (1961).

¹⁰² Albert Darbey, *Anal. Chem.* **24**, 373-8 (1952); A. R. Lavender, T. N. Pullman and D. Goldman, *J. Lab. Clin. Med.* **63**, 299-305 (1964).

100 ml. Development of turbidity occurs slowly. Up to 20 mg. per 100 ml. as sodium citrate does not interfere. Copper, which would interfere, is precipitated by 5,7-dibromo-8-hydroxyquinoline in neutral or faintly acid solution. The complex of cupric ion with EDTA is read in the ultraviolet.

For determination in urine, an excess of nickel sulfate is added and the solution made alkaline with ammonia. Excess nickel is then precipitated by dimethylglyoxime. After filtering off the precipitate, the organic matter in the filtrate is destroyed by digestion with sulfuric acid and hydrogen peroxide. After addition of Chloramine-B, the nickel is determined photometrically (Vol. IIA, pp. 261-5). Serum is deproteinized with nickel sulfate and sodium hydroxide.¹⁰³

Procedure—By bismuth. As a bismuth reagent, dissolve 0.1-0.45 gram of high-purity bismuth in a small volume of 1:15 nitric acid and dilute to 500 ml. with water. As Pyrocatechol Violet reagent, dissolve 0.386 gram of the dye in water and dilute to 1 liter. As a nitrate reagent, mix 1 liter of 20% potassium nitrate solution with 200 ml. of 1:30 nitric acid.

Take a sample containing 0.037-0.52 mg. of EDTA. Add 1.5 ml. of the bismuth reagent and 6 drops of 0.1% 2,6-dinitrophenol in 60% ethanol. Add 1:15 nitric acid until the yellow color of the indicator disappears. Add 1:15 ammonium hydroxide until a faint yellow appears. Add stepwise mixing 10 ml. of the nitrate reagent and 2 ml. of the dye reagent. Dilute to 50 ml. and read at 580 $m\mu$ against a reagent blank.

By cobalt ion. General. Buffer a sample at pH 4 by addition of acetic acid-ammonium acetate buffer, and dilute to 40 ml. Add 5 ml. of 1.3% cobaltous chloride and 2 ml. of 30% hydrogen peroxide. Boil for 10 minutes, cool, and dilute to 50 ml. Read at 535 $m\mu$.

If interfering amounts of copper are present, take a 40 ml. sample. Precipitate with 2 ml. of 1% solution of 5,7-dibromo-8-quinolinol in 2:3 hydrochloric acid. Filter, and add 5 ml. of a buffer for pH 10.8. Dilute to 50 ml. and extract the excess of the precipitating reagent with 10 ml. of ether. This will remove up to 6 mg. of copper per 100 ml. Then buffer to pH 4 and proceed.

Wine. Mix 20 ml. with activated carbon and let it stand in the cold for 2 hours. Filter, and add 5 drops of glacial acetic acid. Add 2 drops

¹⁰³ D. C. Smith and S. L. Tompsett, *J. Clin. Path.* **11**, 365-6 (1958); R. G. Khil and A. P. Kornilova, *Lab. Delo* **10**, 463-6 (1964).

of 2% cobalt nitrate solution and heat at 100° for 15 minutes. Cool to 40° and add 0.5 ml. of 30% hydrogen peroxide. Mix, again heat to 100°, and cool. Read at 530 m μ .

By chromic ion. As an arsenious acid reagent, dissolve 4.95 grams of arsenious oxide and 3.5 grams of sodium hydroxide in about 150 ml. of cold water. Neutralize to phenolphthalein with glacial acetic acid. Slowly add 200 ml. of glacial acetic acid with mixing. Cool and dilute to 500 ml. As arsenious acid-zinc acetate reagent, dissolve 4.13 grams of zinc acetate in arsenious acid reagent and dilute to 250 ml. with that reagent.

Urine. Pipet 2 ml. of sample into each of two tubes. Add 2 ml. of arsenious acid reagent to one and 2 ml. of arsenious acid-zinc acetate reagent to the other. The latter is a corrective blank. Add 2 ml. of 0.55% potassium dichromate solution to each and mix. After 35 minutes at room temperature, read each against its own reagent blank.

By nickel ion. Adjust the pH of a 95-ml. sample to 6.5 and add 15 ml. of 1.33% solution of nickelous sulfate hexahydrate. Let the solution stand for 10 minutes, and if phosphates may be present, add 1.5 ml. of 4.4% calcium acetate. After five minutes, add 5 ml. of concentrated ammonium hydroxide. Any phosphates precipitate and are filtered after 5 minutes. To 60 ml. of filtrate, add 7.5 ml. of 1.5% dimethylglycine in absolute ethanol. Filter after 5 minutes. To 50 ml. of filtrate, add 2.5 ml. of concentrated hydrochloric acid to reduce the pH to 1. After 5 minutes, add 10 ml. of freshly prepared 0.25% aqueous potassium dithiooxalate. After 3 or 4 minutes, add 1.2-1.4 grams of solid sodium acetate crystals, and stir to dissolve. Read at once at 508 m μ .

Plasma or urine. Deproteinize with trichloroacetic acid and neutralize with 8% sodium hydroxide solution. Buffer to pH 11.9 with a glycine buffer. Add an excess of a solution of 1-(2-pyridylazo)-2-naphthol to precipitate. Filter or centrifuge. Extract with chloroform to remove unreacted naphthol reagent. Then proceed as above.

By the ferrous complex of 2,4,6-tripyridyl-s-triazine. As the color reagent, dissolve 0.228 gram of 2,4,6-tripyridyl-s-triazine about 10 ml. of 1:20 hydrochloric acid. Add 50 ml. of 13.6% solution of sodium acetate trihydrate in 1:16 acetic acid. Add 0.0995 gram of ferrous ethylenedia-

mine sulfate and dilute to 500 ml. The pH is 4.5, and excess of the triazine ensures complete complex formation.

To a sample containing 0.2-2 mg. of EDTA or approximately 1.5 times that amount of the sodium salt, add 2 ml. of the color reagent. Mix, and dilute to 25 ml. Read at 593 $m\mu$ against a water blank.

If metals other than the alkali metals are present, read as soon as possible after addition of the color reagent. Then read at intervals of 1 minute for 2-3 minutes and extrapolate back to zero time.

By ferric thiocyanate. As a reagent stock, mix 2 ml. of 50% ammonium thiocyanate solution and 2 ml. of 0.027% ferric chloride hexahydrate solution in 10% acetic acid. Dilute to 20 ml. with water.

To a 1-ml. sample containing 0.02-0.08 millimoles of ethylenediamine tetraacetate or its salts, add 1 ml. of the reagent. Dilute to 3 ml. and read against a water blank at 475 $m\mu$.

By ferric sulfosalicylate. Blood plasma. To 1 ml. of sample containing more than 1 mg. of EDTA, add 1 ml. of 20% solution of sulfosalicylic acid and 2 ml. of water. Centrifuge thoroughly. To 3 ml. of the clear layer, add 1.5 ml. of 0.1% ferric ammonium sulfate solution, slightly acidified with sulfuric acid. Add 0.5 ml. of 16% sodium acetate solution. Dilute with water to 10 ml. and read at 520 $m\mu$ after 15 minutes. For samples containing 0.2-1 mg. of EDTA, reduce the volume of ferric solution to 0.5 ml.

By cupric ion. Take a sample of not over 5 ml. containing 0.125-1.5 mg. of EDTA. Add 4 ml. of 0.25% copper sulfate solution. Mix, and dilute to 25 ml., with 1.42% solution of anhydrous disodium phosphate adjusted to pH 11 with 50% sodium hydroxide solution. This precipitates the unsequestered copper. Set the zero with a solution similarly prepared without EDTA present, and read at 250 $m\mu$.

ETHANOLAMINE AND SERINE

After hydrolysis of the sample, ethanolamine forms a dinitrophenyl derivative upon reaction with dinitrofluorobenzene.¹⁰⁴ Serine may also be determined in the same sample by this method. The ethanolamine

¹⁰⁴ Julius Axelrod, Jules Reichenthal and Bernard B. Brodie, *J. Biol. Chem.* 204, 903-911 (1953); Shoshichi Nojima and T. Nobuyuki, *J. Biochem.* 44, 565-73 (1957).

dinitrobenzene derivative and the serine dinitrobenzene derivative are completely separated by their differential solubilities in organic solvents.

Proteins and lipids are precipitated with colloidal ferric iron, and the nitrogeneous interferences are washed from the precipitate with magnesium sulfate solution. The lipids are extracted into alcohol-ether. Concentrations of 10 micromoles of urea, choline chloride, or inositol do not interfere. Up to 2 micromoles of sodium chloride are tolerated. Alanine, valine, leucine, cysteine-hydrochloride, methionine, phenylalanine, lysine hydrochloride, tyrosine and tryptophan do not interfere. Glycine, cystine and proline show about 10% of the optical density of dinitrophenylserine. Histidine hydrochloride, arginine, oxyproline, aspartic acid and glutamic acid form dinitrophenyl derivatives with the reagent with the same optical densities as that of dinitrophenylserine.

By a method similar to that for the determination of ethylenimine, ethanolamine, a product of the hydrolysis of ethylenimine, forms a red color with 1,2-naphthoquinone-4-sulfonate.¹⁰⁵ The color is extracted into isoamyl alcohol. Beer's law is followed up to 8 ppm. of ethanolamine at 450 mg.

Monoethanolamine in triethanolamine is determined by sodium nitroprusside.¹⁰⁶ The liquid hydrolysate may be chromatographed on Amberlite IR-112 to separate serine and ethanolamine. The fractions are then oxidized with periodic acid to formaldehyde, which is determined as usual by chromotropic acid¹⁰⁷ (Vol. IIIA, p. 252). Ethanolamine and its metabolite, the *S*-oxide are read in the ultraviolet.¹⁰⁸

Starting with a phospholipid, the sample is first hydrolyzed with acid.¹⁰⁹ The fatty acids are extracted with ether. Phosphoric esters in the acid hydrolysate are then decomposed with alkaline phosphatase. After separation by paper chromatography, the ethanolamine is oxidized to formaldehyde. As separated, the glycerol and serine are similarly determined.

Both monoethanolamine and ammonia are determined in the air of

¹⁰⁵ David H. Rosenblatt, Peter Hlinka and Joseph Epstein, *Anal. Chem.* **27**, 1290-3 (1955).

¹⁰⁶ Rene Alquier, *France et ses Parfums* **1**, 30-2 (1958); Makoto Hayashi, Yoshinori Nakajima, Keizo Inoue and Komei Miyaki, *Chem. Pharm. Bull. Japan* **11**, 1200-2 (1963).

¹⁰⁷ Makoto Hayashi, Komei Miyaki and Tsotomu Unemoto, *Chem. Pharm. B-ll* (Tokyo) **8**, 904-7 (1960).

¹⁰⁸ A. Bieder, P. Brunel and L. Mazeau, *Ann. Pharm. Franc.* **21**, 375-87 (1963).

¹⁰⁹ L. W. Wheeldon, M. Brinley and D. A. Turner, *Anal. Biochem.* **4**, 69-80 (1962).

submarines by ninhydrin on silica gel.¹¹⁰ Orthoboric acid removes mono-ethanolamine so that each is determinable by difference.

Sample—*Plasma or red blood cells.* To a 2-ml. sample, add 30 ml. of water and 2.5 ml. of dialyzed iron with 5% ferric oxide and 1.3 ml. of 50% magnesium sulfate solution. Centrifuge for 10 minutes and decant the supernatant liquid. Wash the precipitate by centrifuging three times with 30 ml. of water and 1.3 ml. of 50% magnesium sulfate solution to remove nitrogenous contaminants. Suspend the precipitate in 4 ml. of absolute ethanol. Take up in 4 ml. of 95% ethanol, 4 ml. of ether, and an additional 4 ml. of ether. Dilute to 20 ml. with ether.

Shake for 5 minutes to extract the lipides from the precipitate. Filter and evaporate 10 ml. of the filtrate just to dryness in a stream of air at 50°. Cool, add 0.1 ml. of a 1:24 mixture of hydrochloric acid and 95% ethanol. Add 15 ml. of 30-60° boiling petroleum ether and shake for 10 minutes. Evaporate the petroleum ether from an 8-ml. aliquot containing the phosphatidyl ethanolamine and phosphatidyl serine to dryness in a stream of air.

Dissolve in 1 ml. of 8% sodium hydroxide solution, cover, and heat at 100° for 30 minutes to liberate ethanolamine and serine. Tap the tip of the tube vigorously to dislodge any adhering particles. Heat for 90 minutes at 100°. Add 1 ml. of 1:5 hydrochloric acid and dilute to exactly 3 ml. with water. Clarify by adding a small amount of Hyflo Supercel, and centrifuge. Develop ethanolamine and serine with dinitrofluorobenzene.

Organic extracts. Evaporate the chloroform from a solution of the sample. Add 1 ml. of 24% sodium hydroxide solution, stopper, and heat at 100° for 2 hours. Cool, make the solution acidic with 3 ml. of 1:5 hydrochloric acid. Clarify with a small amount of Celite and activated carbon. Develop ethanolamine and serine with dinitrofluorobenzene.

Procedure—*By dinitrofluorobenzene. Plasma and blood cells.* To 2 ml. of the prepared centrifuged sample, add 0.1 ml. of dinitrofluorobenzene reagent prepared by dissolving 0.1 ml. of dinitrofluorobenzene in 2 ml. of 95% ethanol. Add 1 ml. of 2.5% sodium bicarbonate solution. Stopper. Heat at 75-80° for 1 hour, and cool. Shake with 8 ml. of chloroform for

¹¹⁰ D. D. Williams and R. R. Miller, *Anal. Chem.* **34**, 225-7 (1962).

10 minutes to extract dinitrophenyl ethanolamine. Centrifuge. Dinitrophenyl serine remains in the aqueous phase.

To determine the dinitrophenyl ethanolamine, evaporate the chloroform from 6 ml. of the chloroform extract almost to dryness under a stream of air heating the chloroform with hot water. Add 10 ml. of petroleum ether and 4 ml. of 1:1 hydrochloric acid, and shake for 5 minutes. Read the acid phase at 420 $m\mu$.

Add 2 ml. of the aqueous phase containing the dinitrophenyl serine to 1 ml. of 1:10 hydrochloric acid. Extract the serine by shaking for 10 minutes with 10 ml. of methyl isobutyl ketone. Add 8 ml. of the ketone extract to 4 ml. of 0.4% sodium hydroxide solution, and shake for 5 minutes. Remove the ketone by aspiration. Add 3 ml. of the aqueous phase to 0.5 ml. of 1:5 hydrochloric acid, and read at 420 $m\mu$.

Lipids. To a 1 ml. solution containing 1 micromole of ethanolamine and 1 micromole of serine, add 0.2 ml. of dinitrofluorobenzene reagent as prepared for the previous technic. Add 0.5 ml. of 5% sodium bicarbonate solution. Stopper, and heat at 80° for 1 hour with occasional shaking. Cool, add 10 ml. of xylene, shake vigorously for 5 minutes, and let stand for 10 minutes. The xylene layer contains dinitrophenyl ethanolamine and the aqueous layer contains dinitrophenyl serine. Reserve 8 ml. of the xylene for ethanolamine determination, and discard the remaining xylene layer.

To determine ethanolamine, add 10 ml. of 1:1 hydrochloric acid and 30 ml. of petroleum ether to this xylene layer containing the ethanolamine. Shake, and let stand for 10 minutes. Read the aqueous layer at 420 $m\mu$.

To determine serine, shake the aqueous layer with 8 ml. of xylene for a few minutes. Let stand. Remove the upper layer and discard. Repeat with another 8-ml. portion of xylene. Acidify the aqueous solution with 0.5 ml. of 1:10 hydrochloric acid and extract with three 8-ml. portions of xylene to remove excess reagent. To 2.5 ml. of the aqueous solution, add 0.15 gram of sodium bicarbonate and 5 ml. of water. Read at 400 $m\mu$ for serine.

By 1,2-naphthoquinone-4-sulfonate. Mix 50 ml. of an aqueous solution, containing up to 8 ppm. of ethanolamine, with 10 ml. of 0.138% potassium 1,2-naphthoquinone-4-sulfonate solution and 1 ml. of a 0.05 *M* phosphate buffer at pH 11.7 (Vol. I, p. 175). After 20 minutes, add 15 ml. of isoamyl alcohol and a Teflon-covered magnetic stirring bar.

Stopper, and stir vigorously for 5 minutes. After the layers have separated, decant the isoamyl alcohol and centrifuge to clarify. Read at 450 m μ .

In triethanolamine by sodium nitroprusside. To a 0.2-0.25 gram sample, add 1.8 ml. of water, 1 ml. of 5% sodium bicarbonate solution, 1 ml. of 2% sodium nitroprusside solution, and 1 ml. of acetone. Mix, and compare the color with standards after 15 minutes.

Ethanolamine and its S-oxide. To 5 ml. of plasma or cerebrospinal fluid add 4 grams of sodium chloride and 30 ml. of chloroform. Shake for 4 minutes, then centrifuge thoroughly. Remove the creamy supernatant layer and discard. Mix 25 ml. of the clear chloroform solution with 3 ml. of 1:200 hydrochloric acid, and shake. Centrifuge, and read the clear aqueous layer at 315, 350, or 395 m μ .

NOR HN2, bis(β -CHLOROETHYL)AMINE, AND METHYL
bis(β -CHLOROETHYL)AMINE

Bis(β -chloroethyl)amine hydrochloride and its methyl counterpart alkylate γ -(*p*-nitrobenzyl)pyridine to the quaternary pyridium compound, which is extracted with ethyl acetate from alkaline solution and read at 540 m μ .¹¹¹ Protein is removed by heat denaturation. Acetate buffer increases the sensitivity of the method over phthalate buffer. As low as 0.0005 mg. per ml. can be measured.

Procedure—Tissue homogenates. Take a 1-ml. sample of 10% mouse liver homogenate. Add 0.2 ml. of 1:10 hydrochloric acid and heat at 100° for 3 minutes. Cool and centrifuge for 15 minutes. Decant the supernatant liquid from the precipitated protein and dilute to 3 ml. with water. Cool in ice. Add 1 ml. of 0.2 *M* acetate buffer at pH 4.6 and adjust the pH of the solution to 4.6 with 4% sodium hydroxide solution.

The procedure should now be completed rapidly and largely in the absence of light. Add 0.4 ml. of 5% γ -(*p*-nitrobenzyl)pyridine solution in acetone. Heat for 20 minutes at 100° and cool. Add 2 ml. of acetone, 5 ml. of ethyl acetate, and 1.5 ml. of 10% sodium hydroxide solution. Shake 20 times and centrifuge for 2 minutes. Read the upper layer at 540 m μ .

¹¹¹ Orrie M. Friedman and Eliabu Boger, *Anal. Chem.* **33**, 906-910 (1961).

DIMETHYLAMINOETHYL BENZILATE

This substance can be determined by reaction with acid molybdate.¹¹² Amidopyrine and 2-ethoxybenzamide need not interfere.

Procedure—Tablets. Extract powdered tablets with chloroform for 1 hour and filter. Evaporate 10 ml. of filtrate to dryness at 20°. Add to the residue, 5 ml. of 5% ammonium molybdate in concentrated sulfuric acid. Mix well and read at 610 m μ after 1 hour. The standard used in preparing the calibration curve should contain appropriate amounts of 2-ethoxybenzamide and amidopyrine.

MUSTARGEN, METHYL *bis*(β -CHLOROETHYL)AMINE,
MECHLORETHAMINE

An acid extract of mechlorethamine is determined by its fluorescence at 340 m μ .¹¹³ The method is specific for unchanged mechlorethamine. Chlorimine, the conversion product of mechlorethamine in alkaline solution, interferes to the extent of 10-15%. Hydroxyimine and methyldiethanolamine, which are hydrolysis products, do not interfere. Beer's law is followed up to 0.01 mg. per ml.

Procedure—Plasma or urine. To a 5-ml. ice-cold sample, add 10 ml. of a 9:1 ice-cold mixture of chloroform and methanol. For plasma samples, add 0.1 ml. of 8% sodium hydroxide solution. For urine samples, add a small amount of 2-4% sodium hydroxide solution to bring the pH to approximately 10. To determine the amount of sodium hydroxide to add to urine samples, titrate another portion of the sample to determine the volume of base needed. Keep samples chilled. Shake the solution for 45 minutes at a frequency of 280-300/minute. Centrifuge for 5 minutes and remove the upper aqueous layer.

For urine samples, now shake twice by hand for 1 minute each with 5 ml. of 0.1 *M* borate buffer for pH 10 (Vol. I, p. 177). Centrifuge after each shaking and remove the buffer. For plasma samples, transfer the organic solvent without removing the protein precipitate. Shake for 1 minute each with two 5-ml. portions of 0.1 *M* borate buffer at pH 10. Centrifuge after each shaking and remove the buffer.

Add an aliquot of up to 7 ml. of the solvent layer to 5 ml. of ice-cold

¹¹² H. Siedlanowska, *Farm. Polska* **20**, 160-3 (1964).

¹¹³ L. B. Mellett and L. A. Woods, *Cancer Research* **20**, 518-23 (1960).

1:110 hydrochloric acid and shake on a mechanic shaker for 10 minutes. Centrifuge, and remove the lower organic layer by aspiration. Add 5 ml. of ethylene dichloride and shake. Centrifuge, and remove the ethylene dichloride by aspiration.

Prepare *p*-phenylphenol by adding 3.5 ml. of 2% sodium hydroxide solution and 5 ml. of water to 200 mg. of phenol. Heat until the phenol is dissolved, and dilute to 100 ml. Add a 4.5 ml. aliquot of the hydrochloric acid extract of the sample to 2 ml. of 0.2% *p*-phenylphenol solution, 3 ml. of 0.1 *M* borate buffer at pH 10 and 0.5 ml. of 3.6% sodium hydroxide solution. Heat the solution for 30 minutes at 100° and cool to room temperature. Add 10 ml. of ethylene dichloride and 3 ml. of 2% sodium hydroxide solution. Shake mechanically for 30 minutes. Centrifuge, and remove the upper aqueous layer by aspiration. Shake the ethylene dichloride extract, for 1 minute each, with four successive 5 ml. portions of 4% sodium hydroxide solution. Centrifuge and remove the upper aqueous layer by aspiration after each shaking. Add 7 ml. of the ethylene dichloride extract to 5 ml. of 1:10 hydrochloric acid, and shake for 10 minutes. Centrifuge, and aspirate the lower ethylene dichloride layer. Read the acid extract at an activation wave length of 285 $m\mu$ and a fluorescence wave length of 340 $m\mu$.

PHENYLETHYLAMINES

A general method for phenylethylamine drugs is to read them in the ultraviolet in dilute sulfuric acid solution.¹¹⁴ This method is applicable to *amphetamine*, *methamphetamine*, *mephentermine*, *vonedrine*, *ephedrine*, and *propadrine*, reading the phenylethyl moiety. The method has been further refined for amphetamine by a distillation step (p. 111).

The amine base is extracted from alkaline solution with chloroform, which is washed with 1:350 sulfuric acid. The extracted amine is then read in the acid solution. Interference from impurities is reduced by using a simplified baseline method to measure three characteristic absorbances within the spectral range from 252-262 $m\mu$ at 0.5 $m\mu$ intervals.

Sample—*Tablets or capsules*. Obtain an average weight from 20 samples. Grind 5-20 tablets or capsules to a 60-80 mesh powder. To a sample containing 25-50 mg. of β -phenylethylamine, add 5 ml. of 1:35 sulfuric acid, and swirl gently to allow escape of liberated carbon dioxide,

¹¹⁴ F. A. Rotondaro, *J. Assoc. Offic. Agr. Chemists* **40**, 824-37 (1957).

if present. Test for acidity with litmus paper and add more 1:35 sulfuric acid, if needed. Shake with 20 ml. of chloroform and 7 ml. of 4% sodium hydroxide solution for 3-5 minutes.

In another tube, add 10 ml. of standard β -phenylethylamine solution containing 50-70 mg. in 1:350 sulfuric acid and 0.5 gram of powdered sodium sulfate. Swirl to dissolve. Add 20 ml. of chloroform and 2 ml. of 4% sodium hydroxide solution, and shake for 3-5 minutes. Centrifuge the sample and standard at 1500-1800 rpm for 3-5 minutes. Transfer 10 ml. of the clear lower chloroform layer to a separatory funnel containing 10-15 ml. of 1:350 sulfuric acid. Stopper, shake well for 2-3 minutes, and allow the layers to separate. Drain the clear chloroform layer into 10 ml. of 1:350 sulfuric acid, and shake. Let separate, and discard the chloroform layer containing tablet lubricants and other materials. Wash the two acid layers with 5 ml. of chloroform, discarding the chloroform. Filter the acid solution through cotton. Wash the separatory funnels with several small portions of 1:350 sulfuric acid, and pass the washings through the cotton filter. Dilute the filtrate to 50 ml. with 1:350 sulfuric acid.

Prepare a blank by shaking 25 ml. of 1:350 sulfuric acid with 3-5 ml. of chloroform. Allow the layers to separate, drain, and discard the chloroform. Filter the acid layer through cotton, and dilute to 50 ml. with 1:350 sulfuric acid. Read the sample and blank at 0.5 $m\mu$ intervals in the range of 252-255 $m\mu$ for the first minimum, in the range of 256-258 $m\mu$ for the maximum, and in the range of 260-262 $m\mu$ for a second minimum, using the same slit width for sample and standards. Calculate the absorbance difference between the absorbance at the maximum and the average of the minima as follows:

$$\text{Absorbance difference } (\Delta A) = A_{\max} - 0.5 (A_{\min 1} + A_{\min 2})$$

Calculate the absorptivity differential of the standard produced by 1 mg. per ml. as follows:

$$\text{Absorptivity differential } (\Delta a_{\text{std.}}) = (\Delta A_{\text{std.}} \times 100) / \text{wt. std.}$$

in which

ΔA = absorbance differential for the standard solution

wt. std. = mg. of standard in the aliquot measured

mg. of amine per unit of sample =

$$(\Delta A_{\text{sample}} \times 50 \times 2 \times \text{average weight of unit}) / (\Delta a_{\text{std.}} \times \text{weight sample})$$

AMPHETAMINE, BENZEDRINE, β -PHENYLISOPROPYLAMINE

Amphetamine is distilled from alkaline solutions in the pH range of 7.4-12 and collected in sulfuric acid for reading in the ultraviolet¹¹⁵ (cf. Vol. IV, p. 50). In 0.1 *N* sulfuric acid, amphetamine shows absorption peaks at 263, 257, and 252 $m\mu$, with the peak at 257 $m\mu$ the most prominent. At 257 $m\mu$, amphetamine follows Beer's law up to 100 mg. per 100 ml. The maximum for some possible interferences is shown in Table 14.

TABLE 14. EXTINCTION COEFFICIENTS $E_{1cm}^{1\%}$
FOR AMPHETAMINE AND OTHER ALKALOIDS
IN 0.1 *N* H₂SO₄

	Wavelength	$E_{1cm}^{1\%}$
Amphetamine hydrochloride	257 $m\mu$	11.67
Cocaine hydrochloride	257 $m\mu$	28.75
Cocaine hydrochloride	274 $m\mu$	31.39
Heroin hydrochloride	257 $m\mu$	12.56
Heroin hydrochloride	280 $m\mu$	41.70
Quinine hydrochloride	257 $m\mu$	448.30
Quinine hydrochloride	280 $m\mu$	25.00
Quinine hydrochloride	348 $m\mu$	14.12
Procaine hydrochloride	257 $m\mu$	47.0
Procaine hydrochloride	280 $m\mu$	106.00
Procaine hydrochloride	274 $m\mu$	94.5

In alkaline solution, sugars, starch, mannitol, and magnesium sulfate decompose to produce acetaldehyde, which shows considerable absorption at 257 $m\mu$. Lactose, in particular, interferes. If present in large amounts, these sugars must be separated. Starch may be eliminated by filtration, since it is relatively insoluble in dilute acid. Before distillation, procaine interferes. When distilled, the procaine decomposes to dimethylaminoethanol and aminobenzoic acid, which do not interfere. Other information about interferences appears under phenylethylamines, page 109.

When amphetamine is read at 253.7 $m\mu$, many sympathomimetic and stimulating amines also absorb at this wave length.¹¹⁶ An important application is to determine it as a diluent for illicit cocaine.¹¹⁷

¹¹⁵ W. M. Studebaker and S. M. Wang, *Drug Standards* 27, 41 (1959); Charles Milos, *J. Assoc. Offic. Agr. Chemists* 43, 229-33 (1960).

¹¹⁶ H. Thies and Z. Ozbilici, *Arch. Pharm., Berlin* 295, 715-18 (1962).

¹¹⁷ Charles Milos, *J. Assoc. Off. Agr. Chemists* 43, 229-33 (1960).

Amphetamine is also determined as the methyl orange complex, extractable with chloroform.¹¹⁸

Procedure—*Drugs and narcotics. Lactose absent.* Dissolve a 0.1-gram sample in 1:350 sulfuric acid and dilute to 100 ml. with that acid. To a 45-ml. aliquot, add 25% trisodium phosphate solution until the solution is alkaline to phenolphthalein. Distil. To 25 ml. of the distillate, add 0.06 ml. of concentrated sulfuric acid. Mix thoroughly, and read at 257 m μ .

$$A_{257} \times 85.7 = \text{mg. of amphetamine hydrochloride}$$

If more than 30 mg. of amphetamine are present, repeat the determination. Distil water through the condenser until the volume of distillate is near 50 ml. Add 0.12 ml. of concentrated sulfuric acid, and dilute to 50 ml. with water. Read at 257 m μ .

Appreciable lactose present. Make 50 ml. of the original solution alkaline to litmus with 10% sodium hydroxide solution. Extract with 20, 10, 10, and 5 ml. of chloroform. Wash the combined extracts with 5 ml. of water. Extract the chloroform layer with 20 and 20 ml. of 1:350 sulfuric acid. Proceed as for low lactose content, from "Make 45 ml. alkaline . . ."

*Powdered drugs.*¹¹⁹ Dissolve a sample containing approximately 5 mg. of amphetamine sulfate in 2 ml. of 1:100 hydrochloric acid. Warm, cool, and mix with 3 grams of acid-washed Celite. Pack the sample in a chromatographic column between layers of Celite. Wash the column with 100 ml. of chloroform-saturated water, and discard the washings. Place 10 ml. of chloroform-saturated 1:50 sulfuric acid in the receiver. Elute with 35 ml. of 0.5% solution of triethylamine in water-saturated chloroform. Follow with 70 ml. of water-saturated chloroform. Shake the receiver vigorously and let the phases separate. Read the acid phase against the sulfuric acid as a blank. For this, read at 300-350 m μ and extrapolate to 257 m μ . Ammonia may replace the triethylamine.

Drugs containing large amounts of lactose. To 50 ml. of the original solution in 1:350 sulfuric acid, for the preceding drug procedure, add 10%

¹¹⁸ Robert E. Keller and W. C. Ellenbogen, *J. Pharmacol. Exptl. Therap.* 106, 77-82 (1952).

¹¹⁹ J. E. Moody, Jr., *J. Pharm. Sci.* 52, 791-3 (1963).

sodium hydroxide solution until alkaline to litmus. Extract four times with chloroform, using 20-, 10-, 10- and 5-ml portions. Wash the combined extracts with 5 ml. of water. Extract the chloroform with two 20-ml. portions of 1:350 sulfuric acid. To the aqueous layer, add 5 ml. of 1:350 sulfuric acid, and follow the procedure for drugs, starting at addition of trisodium phosphate. If no interferences are present, cocaine samples containing amphetamine may be analyzed at 274 and 257 $m\mu$. Use the following equations:

$$A_{274} \times 31.86 = \text{mg. cocaine hydrochloride}$$

$$\text{Mg. cocaine hydrochloride} \times 0.0286 = A_{c257}$$

$$(A_{257} - A_{c257}) \times 85.7 = \text{mg. amphetamine hydrochloride}$$

In the presence of methyl cellulose and barbiturate. Grind 20 tablets and weigh 100 mg. of the powder equivalent to 100 mg. of amphetamine phosphate. Take up in 200 ml. of water. Add 5 ml. of 50% sodium hydroxide solution and 5 drops of antifoaming agent. Distil, collecting the distillate in 20 ml. of 1:1800 sulfuric acid. Wash the condenser and adaptor with 1:1800 sulfuric acid, add to the sample, and dilute to 200 ml. Read the amphetamine sulfate at 258 $m\mu$.

METHAMPHETAMINE, *d*-PHENYLISOPROPYLMETHYLAMINE

The method for phenylethylenamines given on page 109 is applicable for direct reading in 1:350 sulfuric acid in the absence of interferences cited there. A technic for separation from interferences by column chromatography is given under amphetamine (p. 112).

N-METHYL- ω -PHENYL-*tert*-BUTYLAMINE, MEPHENTERMINE

The method for phenylethylenamines given on page 109 is applicable for direct reading in 1:350 sulfuric acid in the absence of interferences cited there. A technic for separation from interferences by column chromatography is given under amphetamine (p. 112).

PHENYLPROPYLMETHYLAMINE, VONEDRINE

The method for phenylethylenamines given on page 109 is applicable for direct reading in 1:350 sulfuric acid in the absence of interferences cited there.

EPHEDRINE, 1-PHENYL-2-METHYLAMINOPROPANOL

A method for ephedrine is based on the determination of acetaldehyde formed from it by the reaction of periodic acid.¹²⁰ It can be extracted with hexane for reading in the ultraviolet.

Ephedrine is also effectively determined by Folin's reagent.¹²¹ All substances with a phenolic hydroxyl, *N*-methylephedrine, uric acid, guanine, and oxyanthine react with Folin's reagent. An alternative method is to extract solid samples with 1:120 hydrochloric acid. After chloroform extraction of impurities at pH 4.5-6.5, adjust the pH to 10-13. Extract the ephedrine with chloroform and determine with bromocresol purple.¹²² Ephedrine gives a violet color with ninhydrin at pH 8.¹²³

Aside from three methods cited in Volume III, page 37, the method for phenylethylamines on page 109 is applicable for direct reading in the absence of interferences cited there. For its determination by pyrocatechol, see dibutylamine, on page 137, but read after 1 hour. A technic for separation from interferences by column chromatography is given under amphetamine, on page 112. Phenylpropylamine, phenyramidol and phenylephrine give the same performance.

Ephedrine can be read as a 2:1 chelate with cupric ion.¹²⁴ A similar chelate is formed with phenylephrine. The chelate of ephedrine is extractable with pentanol to separate it from that of phenylephrine, which is not extractable. Pseudoephedrine, *p*-hydroxyephedrine, chloramphenicol, and 2-(diethylamino)ethanol interfere. The reaction probably involves the ethanolamine chain.

Procedure—*By oxidation.* Oxidize a 1-ml. sample containing 0.04-0.15 mg. of ephedrine with 0.5 ml. of a solution containing 0.115% of potassium periodate and 8.4% of sodium bicarbonate. After 10 minutes, add 0.5 ml. of 1% stannous chloride solution in 1:47 hydrochloric acid and 0.1 ml. of 5% *p*-hydroxybiphenyl solution in glacial acetic acid.

¹²⁰ Alf Wickstrom and Bjarne Salvesen, *Medd. Norsk. Farm. Selskap* **14**, 97-104 (1952); Lester Chafetz, *J. Pharm. Sci.* **52**, 1193-5 (1963).

¹²¹ Naokazu Sakata, Tancyoshi Yu, Yoshimi Okada and Hiroko Urabe, *Nippon Kagaku Zasshi* **77**, 256-8 (1956).

¹²² Denichi Ikeda, Hiroko Urabe and Fumiko Yaku, *Yakugaku Kenkyu* **32**, 729-36 (1960).

¹²³ Rihci Fujimoto and Shinsuke Ose, *Yakugaku Zasshi* **79**, 371-4 (1959); N. S. Sekhon, R. N. Dar and Jaishi Ram, *Indian J. Pharm.* **26**, 174-5 (1964).

¹²⁴ B. Salvesen and S. A. Eikill, *Med. Norsk. Farm. Selsk.* **26**, 91-100 (1964).

Slowly add 18 ml. of concentrated sulfuric acid at 0°. After 4 hours, read at 575 m μ .

Alternatively, mix 5 ml. of a sample containing about 2% of ephedrine with 1 ml. of saturated sodium bicarbonate solution. Add 0.5 ml. of 2% sodium iodate solution. After 10 minutes, add 1-2 drops of concentrated hydrochloric acid, and mix. Shake with 20 ml. of hexane and filter the organic layer. Read at 242 m μ .

By Folin's reagent. Prepare an alkaline copper reagent containing 50 ml. of 0.4% sodium hydroxide and 2% of sodium carbonate, to which is added 1 ml. of a solution containing 0.5% of copper sulfate pentahydrate and 1% of sodium tartrate.

To 0.5 ml. of sample containing 0.2-2 mg. of ephedrine, add 2.5 ml. of the alkaline copper reagent. Mix, and let stand for 20 minutes. Add 0.5 ml. of Folin's reagent (Vol. III, p. 116) at half strength. Mix, and read at 660 m μ after 30 minutes against a reagent blank. Alternatively, read at 770 m μ .¹²⁵

By ninhydrin. An appropriate sample contains about 0.125 gram of the hydrochloride in 1:100 hydrochloric acid. Adjust the pH of the sample to 4-5 and dilute to 500 ml. Mix 2 ml. with 1 ml. of 0.5% potassium hydroxide solution. Add 5 ml. of 0.25% ethanolic solution of ninhydrin. Dilute to 10 ml. with water and heat at 100° for 15 minutes. Cool, dilute with water to 50 ml., and read at 570 m μ .

As the copper chelate. Mix 1-15 ml. of sample containing 1-10 mg. of ephedrine with excess of a 2.5% solution of cupric nitrate trihydrate. Add 4% sodium hydroxide solution to adjust to pH 12. After 10 minutes, filter through sintered glass and read at 520 m μ .

DOPAMINE, 3-HYDROXYPHENYLETHYLAMINE, 3-HYDROXYTYRAMINE

The determination of dopamine consists of first oxidizing with manganese dioxide to red 2,3-dihydroindole-5,6-quinone, which is not fluorescent. Under oxygen-free conditions, the latter is rearranged to 5,6-dihydroxy-indole by ultraviolet radiation¹²⁶ or by alkaline zinc sulfite.¹²⁷

¹²⁵ Takeshi Niwase, Hisanori Nishimura and Teruo Tonegawa, *Kagaku to Sôsei* **10**, 116-19 (1957).

¹²⁶ Arvid Carlsson and Bertil Waldeck, *Acta physiol. Scand.* **44**, 293-8 (1958); cf. Fritz Bischoff and Adolfo Torres, *Clin. Chem.* **8**, 370-7 (1962).

¹²⁷ Venkko J. Uusipää, *Ann. Med. Exp. Biol. Fenniae* (Helsinki) **41**, 194-201 (1963).

The method is closely analogous to methods for adrenaline and noradrenaline, but the reaction takes place less readily. After the rearrangement, the fluorescence is effectively stabilized for an hour by adjusting to pH 5 with acetic acid. The fluorescence developed from adrenaline and noradrenaline as adrenolutine or noradrenolutine is at noninterfering wave lengths.

An alternative is to oxidize with iodine and carry out the molecular rearrangement.¹²⁸ The 5,6-dihydroxyindole can be concentrated by adsorbing on alumina. It is then recovered by dilute acetic acid and its fluorescence is read at pH 5.3. For separation of adrenaline and noradrenaline, dopamine, and methoxytyramine by chromatography, see page 161.

Procedure—Tissue extract. Adjust the pH of a 10-ml. sample, in 1:5 hydrochloric acid, to 6.4 by addition of 69% potassium carbonate solution. As a buffer, add 1 ml. of 1:16 acetic acid to 50 ml. of 13.6% solution of sodium acetate trihydrate. Add 2.5 ml. of this buffer and dilute to 20 ml. with water. If adrenaline and noradrenaline are to be determined at the same time, reserve a 4-ml. portion for each. To the remaining 12 ml., add 0.1 gram of manganese dioxide and shake vigorously for 3 minutes. Centrifuge, then filter the supernatant layer. Transfer 4-ml. portions of the clear filtrate to tubes. Add to one tube, 0.5 ml. of a clear 1.45% solution of zinc sulfite in 12% sodium hydroxide solution. After 5 minutes, add 0.5 ml. of 1:2 acetic acid. Shake at once after adding the reagents. As a blank, use the other tube, reversing the order of addition of the reagents. Activate at 335 m μ and read the fluorescence at 385 m μ .

Urine. Prepare an iodine solution by dissolving 0.254 gram of iodine and 5 grams of potassium iodide in water and diluting to 100 ml. Prepare an alkaline sulfite solution by dissolving 2.52 grams of anhydrous sodium sulfite in 10 ml. of water and diluting to 100 ml. with 20% sodium hydroxide solution. Prepare a sodium sulfite solution by dissolving 2.52 grams of anhydrous sodium sulfite in 10 ml. of water.

Adjust a 60-ml. sample to pH 4 with citric acid, and centrifuge to clarify. Take five 5-ml. samples. Pipet 10 ml. of 1.6% sodium acetate solution at pH 8.5-9 and 5 ml. of water into four 100-ml. beakers. Titrate

¹²⁸ Åke Bertler, Arvid Carlsson, Evald Rosengren and Bertil Waldeck, *Kgl. Fysiograf. Sällskap, Lund, Förh.* 28, 121-3 (1958); B. D. Drujan, T. L. Sourkes, D. S. Layne and G. F. Murphy, *Can. J. Biochem. and Physiol.* 37, 1153-9 (1959); Fritz Bischoff and Adolfo Torres, *Clin. Chem.* 8, 370-7 (1962).

one urine sample plus 10 ml. of the sodium acetate solution and 5 ml. of water with 2% sodium hydroxide solution to pH 8.8, using 6 drops of 1% ethanolic phenolphthalein solution. Add sodium hydroxide in the amount indicated by the titration to the other four 10-ml. samples, and add 0.002 mg. per ml. of dopamine standard in 1:10,000 hydrochloric acid to two of the urine aliquots.

The chromatographic tube consists of 1.8×8 -cm. tubing used as a reservoir joined to 0.7×10 -cm. tubing for the alumina column, constricted at the outlet. The collecting receivers consist of 50-ml. graduated cylinders with double-hole stoppers, one hole for the bottom of the adsorption tube and the other for the water aspirator.

Prepare a column by pouring 1 gram of alumina into a water-filled column stopped with a glass wool plug. Stir with a glass rod. Rinse with two 10-ml. portions of water, using suction. Rinse the column with 5 ml. of the sodium acetate solution, using suction. Discard the washes. Pour the acetate solution into the urine sample and add the mixture immediately to the column, adjusting the flow rate to 2 drops per second. If the flow slows, rake the surface of the alumina. Wash with two 5-ml. portions of sodium acetate solution and disconnect the vacuum. Save the solution collected for column pH measurement.

Place a new collecting cylinder at the bottom of the tube, and add 10 ml. of 1.2% acetic acid to the column. Stir up the alumina vigorously with a glass rod and elute by gravity. Repeat with another 10 ml. portion of acetic acid. Detach the column when 20 ml. of eluate is collected. Using a pH meter, adjust the eluate to pH 6.4-6.6 with 20% sodium hydroxide solution. Divide the contents of each cylinder into two 15-ml. centrifuge tubes and centrifuge for 2 minutes. Pipet one 4-ml. sample from each centrifuge tube into each of eight 15-ml. centrifuge tubes, four labelled A and four B.

Add 0.05 ml. of the iodine solution to each tube. After 3 minutes, add 0.5 ml. of alkaline sodium sulfite to each B tube and 0.5 ml. of 18% sodium hydroxide solution to each A tube. The omission of sulfite during rearrangement of the indole in the A tubes, which serve as blanks, results in the destruction of the fluorescent derivative of dopamine. After 3 minutes, add 0.6 ml. of 30% acetic acid to all tubes to bring the pH to 5.3. Add 0.05 ml. of water to each B tube and 0.05 ml. of sodium sulfite solution to each A tube. Read the fluorescence in 5 minutes and in 20 hours, using 320 $m\mu$ for activation and 375 $m\mu$ for reading. Subtract the reading of the A tubes from the corresponding B tubes.

Urine. Alternative method. Follow the procedure under catecholamines, starting with "Alternatively" and ending with "Elute the adsorbed catecholamines with 3.5 ml. of 1:16 acetic acid by shaking for 15 minutes. Centrifuge and decant the eluate." Prepare a buffer for pH 6 by adjusting a 13.6% solution of sodium acetate trihydrate to pH 6 with 1:15 acetic acid.

Dilute an aliquot of the eluate to 1 ml. with 1:67 acetic acid. As a blank, use 1 ml. of 3% acetic acid solution. Add 2 ml. of pH 6 buffer. Oxidize by adding 0.5 ml. of 0.11% iodine solution. After 3 minutes, stop the reaction with 0.5 ml. of 18% sodium hydroxide solution containing 12.6 grams of anhydrous sodium sulfite. After 3 minutes, add 1 ml. of 2:3 hydrochloric acid and let stand for 45 minutes to allow for the decrease in interfering fluorescence. Activate at 330 m μ and read the fluorescence at 375 m μ .

CATECHOLAMINES

Some catecholamines, adrenaline, noradrenaline and dopamine are measured by their fluorescence after oxidation by iodine.¹²⁹ This has been given specifically for dopamine above. In this oxidation, light is necessary for the conversion of noradrenochrome to noradrenolutine. The time required for the maximum development of fluorescence is directly proportional to the intensity of the light. With an intensity of 40 foot-candles, it requires over 45 minutes, but at 8400 foot-candles, it is reached in 2 minutes.¹³⁰

In urine samples, the catecholamines are extracted and concentrated by passage through alumina. At pH 5, catecholamines are oxidized by iodine to the corresponding aminochromes, which are in turn converted to bisulfite derivatives by reaction with sodium bisulfite and read in the ultraviolet at 360 m μ . Sodium acetate is added to enhance the stability of the aminochromes during oxidation.¹³¹ A low pH is used for the sample.

¹²⁹ Roy B. Johnson, *J. Lab. Clin. Med.* **51**, 956-63 (1958); A. Giotti and J. Ingianna, *Arch. ital. sci. farmacol.* **9**, 502-16 (1959); B. D. Drujan, T. L. Sourkes, D. S. Cayne and G. F. Murphy, *Can. J. Biochem. and Physiol.* **37**, 1153-9 (1959); V. V. Men'shikov, *Lab. Delo* **7**, 18-21 (1961); H. G. Kmauff and G. Veerbeek, *Klin. Wochschr.* **40**, 18-23 (1962); S. Brunjes and D. Wybenga, *Clin. Chem.* **9**, 626-30 (1963); G. Ritzel and W. A. Hunzinger, *Klin. Wochschr.* **41**, 419-23 (1963); E. Mantel, *Z. Med. Labortech* **4**, 137-44 (1963).

¹³⁰ L. Chin, A. L. Picchioni, and R. F. Childs, *J. Pharm. Sci.* **52**, 907-9 (1963).

¹³¹ M. Jane Oesterling and Rose L. Tse, *Am. J. Med. Tech.* **27**, 112-24 (1961).

since the catecholamines are stable only in an acid medium. An alternative oxidizing agent is ferricyanide.¹³²

By complex chromatography, not only catecholamines but their metabolites are separated.¹³³ The columns are alumina at pH 6.1, Amberlite CG-50 and Dowex 50-X8. The technic applies to adrenaline, noradrenaline, dopamine, 3,4-dihydroxyphenylalanine (dopa), 3-*O*-methyladrenaline, 3-*O*-methylnoradrenaline, and 3-methoxytyramine. The autoanalyzer has been applied.¹³⁴ Either ethylenediamine¹³⁵ or 3-mercaptopropionic acid¹³⁶ improve the time-stability of the fluorescence at 405 and 436 m μ .

Condensation of noradrenaline with formaldehyde in a nearly anhydrous medium gives an intensely fluorescent compound.¹³⁷ Adrenaline is separated from protein on dry Sephadex equilibrated with 0.01 *M* acetic acid, eluted with 1:16 acetic acid, and concentrated.¹³⁸ Suitable wave lengths for reading the fluorescence of adrenolutine and noradrenolutine are 335 m μ and 385 m μ , respectively.¹³⁹

An interesting technic is to adsorb catecholamines by stirring with alumina in the presence of EDTA with slow addition of sodium hydroxide solution. Thereafter, centrifuge the precipitate and elute with dilute sulfuric acid. For total amines as noradrenaline, oxidize with manganese dioxide at pH 6.5. Add ascorbic acid and sodium hydroxide solution, and read.¹⁴⁰ When catecholamines in a dried film are exposed to formaldehyde vapor, the condensation forms 1,2,3,4-tetrahydroisoquinidines, which rearrange.¹⁴¹ Primary amines with phenolic groups in the 3 and 4 positions fluoresce intensely.

¹³² N. A. Small, *Clin. Chim. Acta* **8**, 803-6 (1963); Aaron H. Anton and David F. Sayre, *J. Pharmacol. Exptl. Therap.* **138**, 360-75 (1963).

¹³³ D. T. Masuoka, W. Drell, H. F. Schott, A. F. Alcaraz and E. C. James, *Anal. Biochem.* **5**, 426-32 (1963).

¹³⁴ R. J. Merrills, *ibid.* **6**, 272-82 (1963).

¹³⁵ U. S. von Euler and F. Lishajko, *Acta Physiol. Scand.* **51**, 348-56 (1961).

¹³⁶ J. F. Palmer, *J. Pharm. Pharmacol.* **15**, 777-8 (1963); *West Indian Med. J.* **13**, 38-53 (1964).

¹³⁷ S. Roston, *U.S. Army Med. Research Lab.*, Rept. No. 295, 12 pp. (1957); Ake Bertler, Arvid Carlsson, and Evald Rosengren, *Clin. Chim. Acta* **4**, 456-7 (1959); Axel Randrup, *Clin. Chim. Acta* **6**, 584-6 (1961); B. Falck and A. Torp, *Med. Exptl.* **5**, 429-32 (1961); *ibid.* **6**, 169-72 (1962).

¹³⁸ C. S. Marshall, *Biochim. Biophys. Acta* **74**, 158-9 (1963).

¹³⁹ G. Ritzel and W. A. Hunzinger, *Klin. Wochschr.* **41**, 419-23 (1963); cf. R. M. Antognetti and S. Nava, *Boll. Soc. Ital. Biol. Sper.* **38**, 1176-8 (1962).

¹⁴⁰ R. Atkinson and N. A. Wynne, *J. Pharm. Pharmacol.* **14**, 794-7 (1962).

¹⁴¹ B. Falck, N. A. Hillarp, G. Thieme, and A. Torp, *J. Histochem. Cytochem.* **10**, 548-54 (1962).

As a method of separation, epinephrine and norepinephrine are sorbed on an alumina column. Then metanephrine and normetanephrine are separated by an Amberlite CG-50 column.¹⁴²

For further details of separation of adrenaline and noradrenaline, dopamine and methoxytyramine by chromatography, see page 161.

Procedure—*Total adrenaline, noradrenaline and dopamine. Urine.* To prepare the iodine solution, dissolve 12.7 grams of iodine in 150 ml. of water containing 18 grams of potassium iodide, allowing several hours for dissolving. Dilute to 1 liter with water. To prepare the sodium thiosulfate reagent, dissolve 12.41 grams of sodium thiosulfate and 50 mg. of sodium carbonate in water and dilute to 1 liter.

Prepare a chromatographic column from an ordinary 50 ml. buret or from glass tubing 11 mm. in internal diameter to which a wide-bore, well-greased stopcock is attached. Place a small glass wool plug just above the stopcock to support the resin. Fill the column and stopcock bore, and tip with water so that no air bubbles remain. Let 3 grams of 150-200 mesh Amberlite CG-50 swell in water for 15 minutes. Transfer the slurry of resin to the column and keep a level of fluid at least 1-2 mm. above the resin when not in use.

With a drip rate of 1-2 ml. per minute, add the following reagents in the order listed, allowing each to drip through until the meniscus just disappears before adding the next: (1) 35 ml. of 1:35 sulfuric acid; (2) 25 ml. of water; (3) 35 ml. of 4% sodium hydroxide solution; and (4) 25 ml. of water. After 1 hour, to allow for the swelling of the resin caused by the alkali, add 25 ml. of 0.4 *M* phosphate buffer at pH 6, dripping through as before.

The samples are 90-minute urine specimens or 200 ml. of urine, whichever is the lesser. One filtered specimen is run as is, another after hydrolysis. The one without hydrolysis gives the free amines, the one after hydrolysis both free and conjugated amines.

To hydrolyze the urine, adjust a filtered sample to pH 1.5 with 1:3 hydrochloric acid. Heat at 100° for 20 minutes, making sure that the level of the urine is below the level of the water in the bath. Remove and immerse in an ice bath with stirring for rapid cooling.

Adjust the hydrolyzed and unhydrolyzed portions of the filtered sample to pH 5.5 by dropwise addition of 4% sodium hydroxide solution with constant stirring. At this stage, take care that the pH does not go above 6, since the catecholamines are destroyed in an alkaline medium.

¹⁴² S. Brunjes, D. Wybenga and V. J. Johns, Jr., *Clin. Chem.* **10**, 1-12 (1964).

Use bromocresol purple as the indicator, checking the final adjustments with a pH meter.

Add the unhydrolyzed sample to the resin and allow it to pass through at a rate of 1-2 ml. per minute. To prepare an automatic setup for this step, use a long-stem 500-ml. separatory funnel with a stem extending into the column for 5-6 inches. With the stopcock of the separatory funnel closed, pour the urine into the funnel and close the top with a tightly fitting stopper to prevent air from entering. Open the stopcock of the funnel slowly and allow the urine to run into the column. The flow of urine will stop automatically when the level of urine in the column is above the tip of the funnel. Air enters only through the tip of the funnel, thus regulating the flow. After all the urine has passed into the column, remove the separatory funnel and add 25 ml. of water to the column. Discard all effluents.

To elute the catecholamines, add 10 ml. of 1:16 sulfuric acid, followed by 15 ml. of 1:3500 sulfuric acid. Collect the eluate in a flask containing 0.5 ml. of 1:35 sulfuric acid. Regenerate the column and similarly isolate the amines from the hydrolyzed sample.

To 30 ml. of eluate at 30°, add 0.4 gram of sodium acetate. Add 4% sodium hydroxide solution dropwise with continuous stirring to adjust the pH to 5.

To 2-ml. portion of the solution in a water bath at 30°, add 0.4 ml. of iodine solution to oxidize. Stop the oxidation exactly 15 minutes later by addition of 0.9 ml. of sodium thiosulfate solution to remove excess iodine. A pink color indicates large amounts of catecholamines. Add 3-6 mg. of sodium bisulfite and mix thoroughly. Prepare a blank with 2 ml. of eluate diluted with 1.3 ml. of water.

Read at 360 and 450 $m\mu$ after 30-60 minutes after addition of sodium bisulfite, or at 360 and 420 $m\mu$ at 18-24 hours after addition of bisulfite. If the 360 $m\mu$ reading exceeds the 420 $m\mu$ or 450 $m\mu$ reading by as much as 0.045 units, the shape of the absorption curve between 300 and 420 $m\mu$ must be determined. If an atypical shape is found for this curve, it would be determined by the individual having taken medication that interferes with this test, such as phenyl-azo-diamine-pyridine or hydroxy-chloroquine.

Calculate as follows:

$$C = D \times 16.5 \times \frac{3.3}{2.0} \times \frac{v}{h} = 27.2 \times D \times \frac{v}{h}$$

in which C is micrograms of catecholamines per hour expressed as norepinephrine equivalents; D is $L_{360} - L_{450}$ (30-60 minutes after

bisulfite) or $L_{360} - L_{420}$ (18-24 hours after bisulfite); v is the final volume of eluate in ml.; and h is the time interval in hours representing the volume of urine used. For conjugated amines, subtract the value for the unhydrolyzed sample from that for the hydrolyzed sample.

By fluorescence. Urine. To prepare the sodium acetate solution, dissolve 16.4 grams of anhydrous sodium acetate in water and dilute to 1 liter. Adjust the pH to 8.5 with a saturated sodium carbonate solution. To prepare the acetate buffer for pH 6.3, add 4 ml. of 6% acetic acid and 0.8 ml. of 10% sodium hydroxide solution to 86 ml. of 8.2% sodium acetate solution. When 1 ml. of this solution is mixed with 0.2 ml. of 1:350 sulfuric acid, the pH should be 6. To prepare the sodium ascorbate, mix 9 parts of 20% sodium hydroxide solution with 1 part of 2% ascorbic acid solution within 1-2 minutes before use. To 2.5 grams of alumina, add 5 ml. of 8.2% sodium acetate solution and stir. Add the slurry to a 1.4×3 -cm. column equipped with a stopcock.

Collect 24-hour samples in bottles containing 10 ml. of 1:1 hydrochloric acid and 2 grams of oxalic acid crystals. Refrigerate the sample at once. Measure the total volume and adjust to 1500 ml. with water. Filter and take a 150-ml. aliquot. Adjust the aliquot to pH 1.5-1.8 with 1:1 hydrochloric acid and divide into three 50-ml. portions. One portion is used to determine the recovery after addition of 1 ml. of 0.001% norepinephrine solution.

Heat the 50 ml. solutions at 100° for 15 minutes and cool under running water. To each, add 30 ml. of acetate buffer at pH 8.5 and adjust the pH to 8.5 by small additions of 25% sodium carbonate solution. Immediately add each to an alumina column and allow to pass through rapidly. Do not allow the columns to run dry. Wash the alumina first with 10 ml. of acetate buffer at pH 8.5, then with 50 ml. of water. Add 1:139 sulfuric acid to the column and allow to pass through at a slower rate. When the eluate becomes acid to bromothymol blue paper, collect 10 ml.

Read the fluorescence with a Farrand photofluorometer with the following filters: primary Corning Nos. 5860, 5113 and 3389 and secondary Corning Nos. 3384 and 5433. The filters represent activation at 365, 400 and 436 $m\mu$ and reading at 510 $m\mu$.

Alternatively, collect the sample in 5 ml. of glacial acetic acid or concentrated hydrochloric acid for a 24-hour specimen. Store in the refrigerator. To a 10-ml. aliquot, add 2.5 ml. of 10% ethylenediamine tetraacetic acid solution, 0.5 gram of neutral alumina of chromatographic

grade, and 1 drop of 1% phenolphthalein solution. Shake, and adjust the pH to 8 with 5% sodium hydroxide solution with constant swirling. Shake again for a total shaking period of 5 minutes. Allow the alumina to settle and remove the supernatant layer. Shake the suspension of moist alumina with 2.5 ml. of 1.6% sodium acetate solution for 10 minutes. Discard the supernatant layer and repeat with washing of the alumina with water.

Elute the adsorbed catecholamines with 3.5 ml. of 1:16 acetic acid by shaking for 15 minutes. Centrifuge, and decant the eluate.

To determine adrenaline and noradrenaline, dilute an 0.8-ml. aliquot of the eluate to 1 ml. with water. Add 2 ml. of pH 6 buffer and mix. Oxidize with 0.5 ml. of 0.011% iodine solution for 3 minutes. Stop the reaction with 0.5 ml. of 0.08% sodium thiosulfate solution. Bring about lutein formation by adding 1 ml. of 18% sodium hydroxide solution containing 2 mg. of ascorbic acid. Let stand for 15 minutes and read the fluorescence at 510 $m\mu$ with activation at 420 $m\mu$.

To determine adrenaline only, use 0.1 N Sorensen's glycine-hydrochloric acid buffer for pH 3 in place of the buffer for pH 6 in the oxidation step.

To determine dopamine, dilute an aliquot of the eluate to 1 ml. with 1:35 acetic acid. Add 2 ml. of the buffer for pH 6. Oxidize as before with 0.5 ml. of 0.011% iodine solution for 3 minutes. Stop the reaction with 0.5 ml. of 1% sodium hydroxide solution containing 0.25% of anhydrous sodium sulfite. After 3 minutes, add 1 ml. of 1:1 hydrochloric acid. Let it stand for 45 minutes to cut down on interfering fluorescence, and activate at 330 $m\mu$ for reading at 375 $m\mu$.

As another alternative technique¹⁴³ for free and bound adrenaline and noradrenaline in urine, precipitate calcium and magnesium at pH 6-6.5 by addition of saturated ammonium oxalate solution. Adsorb catecholamines on an alumina column at pH 8.2-8.3 in the presence of phenolphthalein and a 4% solution of sodium hydroxide. Elute with 20 ml. of 1:86 acetic acid.

To 2 ml. of eluate, add 1 ml. of 10.25% anhydrous disodium phosphate to adjust the pH to 6.5. Add 0.3 ml. of 0.127% iodine solution. Stir for 30 seconds and add 2 drops of 2.5% sodium thiosulfate pentahydrate solution. After 5 minutes, add 0.3 ml. of 1% ascorbic acid solution and 1 ml. of 20% sodium hydroxide solution. Adjust to 10 ml. with water. Centrifuge, and read the fluorescence derived from adrenaline and noradrenaline.

¹⁴³ P. A. Kaliman, *Voprosy Med. Khim.* 8, 407-11 (1962).

To another 2 ml. of eluate, add 0.8 ml. of 2.72% sodium acetate to adjust the pH to 4.4. Add reagents as before. The fluorescence is from adrenaline alone.

For bound catecholamines, reflux 25 ml. of urine with 1.5 ml. of concentrated hydrochloric acid for 20 minutes. Then determine as before.

METANEPHRINE, α -[(METHYLAMINO)METHYL]VANILLYL ALCOHOL
AND
NORMETANEPHRINE, α -(AMINOMETHYL)VANILLYL ALCOHOL

These two major metabolites of catecholamines are determined in body fluids and tissue.¹⁴⁴ The steps are hydrolysis at pH 1, passage through alumina to remove interfering substances such as epinephrine and norepinephrine, desalting, incubation with sulfatase, adsorption of the amines on cation-exchange resin and, after elution, application of the fluorescence by the trihydroxyindole method. The method will determine 0.02 and 0.2 microgram, respectively, of metanephrine and normetanephrine.

Procedure—Urine. To prepare the Amberlite CG-50, Type 1, soak overnight in 1:5 hydrochloric acid. Wash by decantation and stir for 30 minutes with 10 volumes of 0.8% sodium hydroxide solution. Wash. Repeat the acid and alkaline treatments three times. Finally, stir the resin in the sodium form with *M* acetate buffer for pH 6. Adjust the pH of the mixture to 6 with dilute acetic acid and stir for 2 hours. Adjust the pH from time to time and store in the acetate buffer for pH 6. For use, wash with water and suck dry.

Adjust 25 ml. to pH 1-1.5 and reflux for 15 minutes. Cool and add 2.5 ml. of 1% solution of sodium ethylenediamine tetraacetate and 2.5 ml. of 2% solution of ascorbic acid. Adjust the pH to 8.4 with ammonium hydroxide and centrifuge. Pass through a column of 0.7 grams of alumina under a pressure of about 1 pound per square inch. Raise the pH of the filtrate to 10.3. Place in the center compartment of the electro dialyzer (Fig. 3) and start to desalt at a current of 1 ampere. Continue until the current drops to 0.4-0.5 ampere, usually 20-30 minutes. If the pH as checked with indicator paper drops materially, add a drop of concentrated ammonium hydroxide. The sample becomes heated and may boil.

¹⁴⁴ Elizabeth R. Smith and H. Weil-Malherbe, *J. Lab. Clin. Med.* **60**, 212-23 (1962). Cf. A. Bertler, A. Carlsson, and E. Rosengren, *Acta Physiol. Scand.* **273-93** (1958).

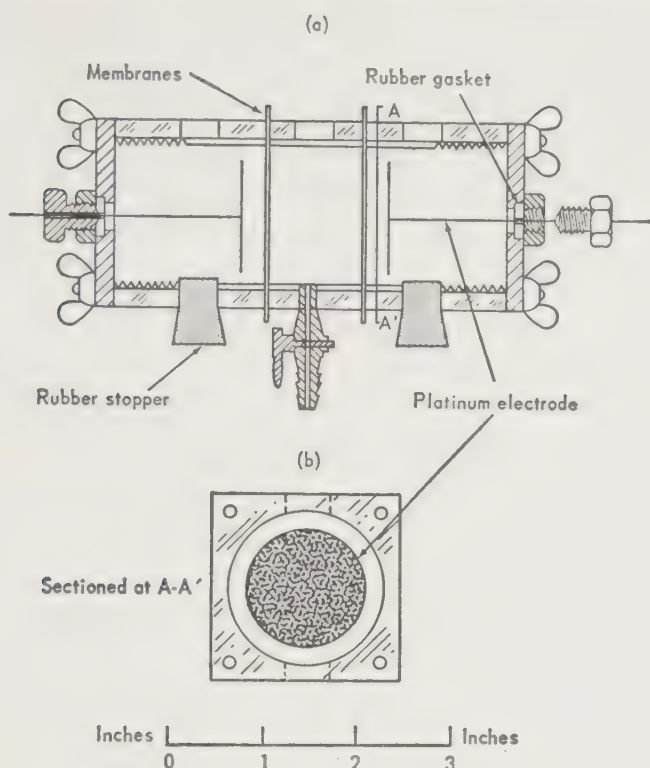


FIG. 3.

Electrodialyzer: (a) longitudinal section; (b) cross-section

Adjust the desalted urine to pH 5.5 with 1:16 acetic acid. Add 1% by volume of a sulfatase preparation (Glusulase, Endo Laboratories) and incubate at 37° for 24 hours. Adjust the pH to 6 with ammonia and pass through a column of 0.5 gram of the pretreated Amberlite CG-50, Type 1. Wash the column with 15 ml. of water. Elute with 5 ml. of *M* formic acid at not over 0.5 ml. per minute. Complete the elution with 2 ml. of water. Divide the eluate into two parts for the determinations that follow.

Metanephrine. As a 0.1 *M* citrate buffer for pH 3, dissolve 2 grams of citric acid in about 80 ml. of water. Add 20% sodium hydroxide solution dropwise to adjust to the desired pH and dilute to 100 ml. It is stable for a week.

Dilute the sample to 5 ml. and add 0.5 ml. of 0.5% zinc sulfate heptahydrate solution as catalyst. Add 0.1 ml. of 0.25% potassium ferrieyanide solution. After 5 minutes, add 0.5 ml. of a fresh mixture of 9 volumes of 20% sodium hydroxide solution and 1 volume of 2% ascorbic acid solu-

tion to rearrange the indole to the fluorescent form. Read the fluorescence within 20 minutes, exciting at 405 m μ and reading at 512 m μ . Compare with standards of 0.25 and 0.5 microgram of metanephrine in 1:1000 hydrochloric acid diluted to 3.5 ml. with a mixture of the 0.1 *M* citrate buffer and *M* formic acid, final pH 2.6. Subtract a reagent blank from each.

Metanephrine and normetanephrine. As a 0.5 *M* buffer for pH 7.9, mix 60.5 grams of *tris* (hydroxymethyl)aminomethane with 316 ml. of 1:10 hydrochloric acid and dilute with water to 1 liter. Adjust the pH of a portion of the prepared sample to 7.9 and dilute to 6 ml. with the *tris* buffer. Add 0.1 ml. of 0.25% iodine solution. After 5 minutes, add 0.5 ml. of the alkaline ascorbate solution used in determination of metanephrine. Read the fluorescence after 1 hour as above. For comparison, use a standard of 0.5 microgram of metanephrine and standards of 0.5 and 1 microgram of normetanephrine, each diluted to 6 ml. with the *tris* buffer for pH 7.9.

An alternative is to correct with a sample blank in which the alkaline ascorbate is added before the oxidizing agent, so that the fluorescent derivatives of metanephrine and normetanephrine are not formed.

Then

$$\text{normetanephrine} = [(0.5e - Mf)/g] \mu\text{g}$$

where *e* is the fluorescence reading when oxidized at pH 8; *f* is the reading of 0.5 μg of metanephrine standard oxidized at pH 8; *g* is the reading of 0.5 μg of normetanephrine standard oxidized at pH 8; and *M* is the μg of metanephrine in the sample oxidized at pH 3.

α -METHYL DOPA ETHYL ESTER

This ethyl ester of α -methyl-3,4-dihydroxyphenylalanine is isolated at pH 4.5 on a powdered cellulose chromatographic column. Thereafter, it is developed with 4-nitro-2-chloro-1-diazo-phenyl- β -naphthylsulfonic acid.¹⁴⁵

Procedure—Prepare a 40 \times 2-cm. chromatographic column fitted to deliver into a volumetric flask. Mix 16 grams of powdered cellulose with about 150 ml. of chloroform. Equilibrate a 0.1 *M* citrate buffer for pH 4.5 to which has been added 0.35% of sodium bisulfite and 0.05% of ethylenediaminetetraacetic acid against *n*-butanol. Add to the chloroform-cellulose mixture with vigorous agitation. Pack the column with this.

¹⁴⁵ Arnold D. Marcus and Joseph D. DeMarco, *J. Pharm. Sci.* 52, 402-3 (1963).

using moderate pressure. Wash the column with 50 ml. of *n*-butanol, which has been equilibrated against the buffer. As it enters the column, add 1 ml. of sample containing 5-10 mg. of the test substance. As it enters the column, wash down the sides with 5 ml. of *n*-butanol and follow with another 5-ml. portion of *n*-butanol.

Add 25 ml. of *n*-butanol saturated with buffer and maintain a constant head. Collect the effluent either fractionally or as a whole. The test substance is concentrated in the portion from 30 to 60 ml. Dilute an aliquot with 1:2 acetic acid to contain 10-15 micrograms of test substance per ml. Mix 5 ml. of this with 5 ml. of phenyl- β -naphthylsulfonic acid in 1:2 acetic acid. Dilute to 25 ml. with 1:2 acetic acid and heat at 35° for 1 hour. Cool rapidly and read at 410 $m\mu$ against a reagent blank.

NOREPHEDRINE, PROPADRINE, PHENYLPROPANOLAMINE

The method for phenylethylamines, on page 109, is applicable for direct reading in 1:350 sulfuric acid in the absence of interferences cited there.¹⁴⁶ A technic for separation from interferences by column chromatography is given under amphetamine, on page 112.

Phenylpropanolamine is read in dilute sulfuric acid after extraction from an alkaline salt solution of the sample with chloroform and re-extraction of the amine into dilute sulfuric acid.¹⁴⁶ Interference from impurities is reduced by using a simplified baseline method that measures three absorbances within the spectral range of 252-262 $m\mu$. Norephedrine is also determined in the ultraviolet after treatment with iodate.¹⁴⁷

Procedure—By oxidation. Urine. Dilute a sample containing about 0.025 mg. of norephedrine to 1 ml. Add 4% sodium hydroxide solution until alkaline to phenolphthalein. Add 1 ml. of 1.5% sodium iodate solution and shake for 10 minutes. Extract with 10 ml. of ethyl ether. Wash the ether extract by shaking for 5 minutes with 5 ml. of 0.4% sodium hydroxide solution. Read the ether layer at 242.5 $m\mu$ maximum and 266 $m\mu$ minimum. Calculate from the difference in these readings.

In the ultraviolet. Tablets or capsules. To prepare the standard, weigh 500-700 mg. of the phenylethylamine salt and dissolve in 1:350 sulfuric acid. Dilute to 100 ml. with 1:350 sulfuric acid and mix well.

¹⁴⁶ Felice A. Rotondoro, *J. Assoc. Offic. Agr. Chemists* **41**, 509-11 (1958).

¹⁴⁷ K. Henrich, D. R. MacDonnell, T. L. Flanagan and P. D. O'Brien, *J. Pharm. Sci.* **50**, 232-7 (1961).

Weigh 20 or more samples and obtain an average weight. Grind to a uniform 60-80 mesh powder. Add a weighed sample to a centrifuge tube containing 3-3.5 grams of sodium chloride and 6-7 glass beads. Dissolve with 5 ml. of 1:35 sulfuric acid and swirl gently to allow the escape of carbon dioxide. Test for acidity with litmus and add more acid if necessary. Shake with 25 ml. of chloroform and 7 ml. of 4% sodium hydroxide solution for 3-5 minutes. To a second centrifuge tube, add 10 ml. of the standard solution and 3-3.5 grams sodium chloride. Swirl gently to dissolve and shake with 25 ml. of chloroform and 2 ml. of 4% sodium hydroxide solution for 3-5 minutes.

Centrifuge at 1500-1800 rpm for 3-5 minutes and withdraw 10 ml. of the clear lower chloroform layer by closing the upper end of a pipet with the finger while lowering the tip through the aqueous layer. Wipe off any drops remaining on the outside of the pipet. Transfer the 10 ml. chloroform portion to a second centrifuge tube containing 25 ml. of 1:350 sulfuric acid and 6-7 glass beads. Repeat shaking and centrifuging as above.

To prepare the acid blank, shake 25 ml. of 1:350 sulfuric acid with 3-5 ml. of chloroform. Allow the layers to separate and discard the chloroform. Filter the acid layer through cotton and dilute the filtrate to 50 ml. with 1:350 sulfuric acid. Read the filtered solutions in 1-cm. cells at 0.5 $m\mu$ intervals in the range of 252-255 $m\mu$ for the first minimum, 256-258 $m\mu$ for the maximum, and 260-262 $m\mu$ for the second minimum, using the same slit width for sample and standard.

Calculate absorbance difference (ΔA) between the absorbance at maxima and the average of the 2 minima:

$$\Delta A = A_{\max} - 0.5 (A_{\min 1} + A_{\min 2})$$

Calculate the absorptivity differential (Δa) produced by 1 gram per liter of the standard amine base or salt:

$$\Delta a_{\text{std.}} = \Delta A_{\text{std.}} \times \frac{100}{\text{wt. std.}}$$

where $\Delta A_{\text{std.}}$ is the absorbance difference for the standard solution; 100 is the ml. standard solution measured; and wt. std. is the mg. standard in aliquot measured.

The mg. of amine per unit of sample is:

$$(\Delta A_{\text{sample}} \times 50 \times 2 \times \text{average wt. of unit}) / (\Delta a_{\text{std.}} \times \text{wt. sample})$$

in which 50 is the volume of sample solution and 2 is the aliquot factor.

Δ -AMINOLEVULINIC ACID

Δ -Aminolevulinic acid, a precursor of porphyrin, is determined by condensation with a carbonyl compound, such as acetylacetone or ethyl acetoacetate, to form a pyrrole. This reacts with *p*-dimethylaminobenzaldehyde to form a red compound.¹⁴⁸ Alternatively, when the acid is mixed with picric acid and an excess of sodium hydroxide, a red-brown color is formed upon acidification with hydrochloric acid.¹⁴⁹ The picric acid method is less sensitive than the method with *p*-dimethylaminobenzaldehyde, but is not subject to as many interferences.

In the determination of Δ -aminolevulinic acid as a pyrrole, there is more interference from amino acids, ammonia, and glucosamine when the acid is condensed with ethyl acetoacetate rather than with acetylacetone. Glucosamine and *N*-acetylglucosamine give color with the reagent, and when present, the acid should be determined by the picrate method. Up to 2 mg. of pyruvic acid, glutamic acid, succinic acid, glucose, and glycine do not interfere. Porphobilinogen gives a red color with the reagent.

Urine samples are run through a Dowex 2 column to eliminate meso-bilirubinogen and porphobilinogen. Thereafter, the Δ -aminolevulinic acid is sorbed on Dowex 50 and eluted with sodium acetate solution after removal of urea with water. Beer's law is followed for 0.05-0.5 micromole of Δ -aminolevulinic acid.

In the determination of Δ -aminolevulinic acid with picric acid, aminoacetone gives the same reaction. Large amounts of keto acids should be removed by chromatography. Up to 0.1 *M* quantities of glucose, threonine, and inorganic phosphate, and 2.5% of trichloroacetate, do not interfere. Porphobilinogen may be present in concentrations less than that of the acid.

Since the interval of time between the addition of sodium hydroxide and the acidification with hydrochloric acid is critical, the reaction is carried out at 0° to extend this time interval. Beer's law is followed up to 0.8 micromole of Δ -aminolevulinic acid and up to 0.5 micromole of aminoacetone. The color fades with both compounds at the rate of 2% per 10 minutes. The color is read at 495 m μ to reduce interference from excess picric acid and eliminate need to remove the excess.

¹⁴⁸ D. Mauzerall and S. Granick, *J. Biol. Chem.* **219**, 435-446 (1956).

¹⁴⁹ L. Shuster, *Biochem. J.* **64**, 101-6 (1956); W. H. Elliot, *ibid.* **74**, 90-4 (1960); T. Malihowska, *Chem. Anal. (Warsaw)* **5**, 1049-54 (1960).

Sample—Urine. Stir Dowex 2-X8 and Dowex 50-X8 in water and decant until the supernatant liquid is clear. To convert the Dowex 2-X8 to the acetate form, wash the resin in a column with 25% sodium acetate solution until the eluate is chloride-free. Wash with water until the eluate is free of sodium acetate. Convert the Dowex 50-X8 to the sodium form by letting it stand overnight with 4% sodium hydroxide solution. Wash until neutral. Treat the resin with 1 volume of 1:2 hydrochloric acid, followed by 6 volumes of 1:5 hydrochloric acid, 6 volumes of 1:110 hydrochloric acid, and water, to convert the resin to the acid form.

The chromatographic column consists of a 0.7×30 -cm. tube with indentations at a level of 10 cm. from the lower end. Make the lower end water-repellent with Beckman Desicote. Place a glass wool plug above the indentations and add a slurry of the resin to a height of 2 cm. Plug with a glass wool stopper. The flow rate should be 3 ml. per 10 minutes.

Add 1 ml. of sample at pH 5-7 to a Dowex 2 column. Add two 2-ml. portions of water and transfer the eluate to a Dowex 50 column. Wash the Dowex 50 column containing the acid and urea with 16 ml. of water to remove urea. Add 3 ml. of 4% sodium acetate solution. Drain the aqueous layer and eluate the aminolevulinic acid with a further 7 ml. of 4% sodium acetate solution. Develop the eluate with *p*-dimethylaminobenzaldehyde and acetylacetone.

Separation of Δ -aminolevulinic acid and aminoacetone. Prepare a column of Amberlite IRC-50. Equilibrate the column with sodium acetate buffer at pH 4.7-5. Wash with water and run the sample at pH 5 through slowly.

The aminoacetone is retained and the Δ -aminolevulinic acid passes through. Wash the column with water and elute the aminoacetone with 1:5 hydrochloric acid. Evaporate the eluate to dryness in vacuo and extract the aminoacetone hydrochloride with absolute ethanol. Filter from the precipitated sodium chloride.

Procedure—By *p*-dimethylaminobenzaldehyde and acetylacetone. To prepare the reagent, dissolve 1 gram of *p*-dimethylaminobenzaldehyde in 30 ml. of glacial acetic acid. Add 8 ml. of 70% perchloric acid and dilute to 50 ml. with acetic acid. The reagent is unstable and should be used on the day it is prepared. To prepare an acetate buffer for pH 4.6, add 57 ml. of glacial acetic acid to 136 grams of sodium acetate trihydrate and make up to 1 liter with water. To the eluate of the sample, which has been passed through Dowex 2 and 50 to remove interferences, add 0.2 ml.

of acetylacetone and dilute to 10 ml. with acetate buffer for pH 4.6. Stopper, heat at 100° for 10 minutes, and cool to room temperature. Add 2 ml. of the reagent and read at 553 $m\mu$ against a reagent blank.

By p-dimethylaminobenzaldehyde and ethyl acetoacetate. Prepare the reagent as in the procedure with acetylacetone. To prepare a phosphate buffer for pH 6.8, mix equal volumes of 6.9% monobasic sodium phosphate and 7% dibasic sodium phosphate.

To a sample containing aminolevulinic acid, add 0.2 ml. of ethyl acetoacetate and dilute to 10 ml. with the phosphate buffer for pH 6.8. Stopper, and heat at 100° for 10 minutes. Mix with an equal volume of reagent and read at 553 $m\mu$ after 5 minutes.

By picric acid. Aminolevulinic acid or aminoacetone in the absence of keto acids. To a 2.5-ml. sample containing 0.05-0.08 micromole of Δ -aminolevulinic acid or 0.05-0.5 micromole of aminoacetone, add 0.25 ml. of a saturated picric acid solution. Cool to 0° and add 0.5 ml. of 20% sodium hydroxide solution at 0°. Mix, and let stand at 0° for 6 minutes for aminoacetone and 14 minutes for Δ -aminolevulinic acid. Add 1.75 ml. of 1:2 hydrochloric acid and warm to room temperature. Read at 495 $m\mu$ after 10 minutes.

In the presence of keto acids. Pass the sample through a 5 \times 0.8-cm. column containing Dowex 50 (H type) and wash the column with 20-bed volumes of water. Elute the amino compounds with 25 ml. of 1:5 hydrochloric acid and evaporate the eluate to dryness in vacuo. Dissolve the residue in a measured amount of water and develop an aliquot with picric acid by the method for keto acids absent.

2-DIETHYLAMINOETHYL BENZILATE, BENACTYZINE

Benzilic acid, a hydrolysis product of benactyzine, is extracted with ether from an acidified solution, leaving the benactyzine in the aqueous layer. After the solution is made alkaline with sodium bicarbonate, the benactyzine is extracted with ether, the solvent removed, and the residue dissolved in ethanolic hydrochloric acid.¹⁵⁰ It is then read at 258.5 $m\mu$. The bacteriostats used in solutions for injection do not interfere. The solution may also be nitrated and read.¹⁵¹

¹⁵⁰ J. P. Jefferies and J. I. Phillips, *J. Pharm. and Pharmacol.* **8**, 907-14 (1956).

¹⁵¹ J. Vacek, *Cesk. Farm.* **10**, 187-8 (1961).

Benaetyzine is precipitated quantitatively from aqueous solution by phosphomolybdic acid, dissolved in acetone, and read at 400-450 $m\mu$.¹⁵² Beer's law is followed for 0.01 mg. per ml. Benaetyzine precipitates bismuth. The precipitate is soluble in acetone for reading.¹⁵³ Accuracy is to 3-4%. It is also read in the visible range in sulfuric acid solution.¹⁵⁴ Beer's law is followed for 0.002-0.005% in 95% sulfuric acid.

Procedure—*In hydrochloric acid. Injection solutions.* Dilute a sample containing 40 mg. of benaetyzine hydrochloride to 10 ml. with water. Add 10 ml. of 1:10 hydrochloric acid and extract with 40 ml. and 25 ml. of ether. Wash the combined ether extracts with two 5-ml. portions of 1:24 hydrochloric acid and add the washings to the original aqueous layer. Discard the ether extracts.

To the aqueous solution, cautiously add with swirling 1.4 grams of sodium bicarbonate. The solution should be alkaline to litmus. Extract immediately with 50 ml., 30 ml., and 15 ml. portions of ether. Wash the combined ether extracts with two 10-ml. portions of water containing a small quantity of sodium carbonate. Filter the ether extract through cotton and remove the solvent by distillation. Dissolve the residue in 100 ml. of cold 1:110 hydrochloric acid in ethanol and read at 258.5 $m\mu$.

Tablets. Weigh 30 tablets and powder finely. To a weighed portion containing approximately 25 mg. of benaetyzine hydrochloride, add 50 ml. of 1:24 hydrochloric acid and mix by swirling. Let stand for 5 minutes. Stopper, shake mechanically for 10 minutes, and filter. Extract a 20-ml. portion of the filtrate following the procedure under solutions, starting with “. . . extract with 40-ml. and 25-ml. of ether.” At the end of the procedure, substitute 25 ml. of 1:110 hydrochloric acid in ethanol for the 100 ml. used with injection solutions.

By phosphomolybdic acid. Drugs. Triturate a solution containing 0.01-0.05 mg. of benaetyzine with 30 ml. of water. Add 0.5 ml. of 1:2 hydrochloric acid and heat at 100° for 5 minutes. Centrifuge. Decant and wash the residue with 10 ml. of water. Centrifuge and wash the residue with 5 ml. of water. Treat the combined extracts with 1 gram of sodium bicarbonate and extract with three 20-ml. portions of ether. Extract the

¹⁵² W. Chiti, *Farmaco Ed. prat.* **15**, 759-63 (1960).

¹⁵³ István Floderer and Valéria Horváthy, *Acta Pharm. Hung.* **30**, 110-17 (1965).

¹⁵⁴ H. Seidlanowska, *Acta Polon. Pharm.* **21**, 33-40 (1964).

combined ether layers with 30 ml. of 1:20 hydrochloric acid. Heat for 5 minutes at 100°. Treat with 1 ml. of concentrated hydrochloric acid and 4 ml. of 20% phosphomolybdic acid solution. After 10 minutes, filter the precipitate. Wash with 10 ml. of 1:10 hydrochloric acid and dissolve in 50 ml. of acetone. Read at 400-450 m μ .

By bismuth precipitation. As reagent, prepare a saturated solution of bismuth subnitrate in 20 ml. of 1:6 sulfuric acid. Add 27.7 grams of potassium iodide in 25 ml. of water. Let stand for 2 days, decant, and dilute to 100 ml. This keeps for 2-3 months. Diluted to one-tenth strength, it decomposes after 3 days.

Tablets. Dissolve a pulverized sample containing 12-15 mg. of benactyzine hydrochloride in 50 ml. of water. Add 0.5 gram of sodium bicarbonate and extract with chloroform. Extract the chloroform solution with 20 ml. of 1:100 hydrochloric acid at once. Heat the extract until dissolved chloroform is evaporated. Dilute the cooled acid solution to 25 ml. with 1:100 hydrochloric acid.

To a 10-ml. aliquot, add 1 ml. of concentrated hydrochloric acid and 5 ml. of the 1:10 dilution of the reagent. The precipitate is a complex with bismuth tetraiodide. Shake for 15 minutes, add 10 ml. of water, and shake again. Filter and wash with water saturated with the precipitate. Dissolve the precipitate in 10 ml. of acetone. Dilute 1 ml. of this to 10 ml. with acetone and read at 500 m μ .

By nitration. Evaporate a solution of the sample in ethanol. Add 0.5 ml. of concentrated nitric acid and after 1 minute 6.5 ml. of concentrated sulfuric acid. Dilute with water and read against a blank after 20 minutes.

In sulfuric acid. Tablets. Add 1 gram of finely ground sample to 5 ml. of water. Add 15 ml. of hot ethanol. Shake for a few minutes, cool, and dilute to 25 ml. with ethanol. Filter, rejecting the first 5 ml. of filtrate. Evaporate the next 10 ml. to about 1.5 ml. and shake with 60 ml. of chloroform for 30 minutes. Separate the chloroform layer and add 7 grams of anhydrous sodium sulfate. Shake for 30 minutes. Evaporate 40 ml. of the chloroform solution to dryness. Take up the residue in 10 ml. of 95% sulfuric acid and read at 498 m μ after 90 minutes.

1-DIETHYLAMINOETHYLAMINE-4-METHYLTHIOXANTHONE,
LUCANTHONE

Lucanthone may be extracted with chloroform or acetone-ether and read in chloroform or hydrochloric acid.¹⁵⁵ The acetone-ether hydrochloric acid method is more accurate.

Procedure—Plasma. Dilute a 2.5-ml. sample to 10 ml. with 1:300 hydrochloric acid and add 1 ml. of chloroform. Shake gently for 2 minutes, let stand for 5 minutes, and add 5 ml. of 0.4% sodium hydroxide solution. Centrifuge, and read the clear chloroform layer immediately against chloroform using a violet filter.

Alternatively, dilute a 2.5-ml. sample to 6 ml. with acetone. Stir, and let stand for 1-2 minutes. Centrifuge. Pour the supernatant liquid into 5 ml. of 4% sodium hydroxide solution. Add 6 ml. of ether and shake for 2 minutes. Decant the ether layer and shake the remaining solution with 1-2 ml. of ether. Treat the combined ether extracts with 1.5 ml. of 1:110 hydrochloric acid. Shake, and dilute the acid layer to 2.5 ml. with 1:110 hydrochloric acid. Centrifuge, and read the acid layer.

PHENYLEPHRINE, 1-*m*-HYDROXY- α -METHYLAMINOMETHYL
BENZYL ALCOHOL

Phenylephrine is determined by coupling the phenol with 4-aminoantipyrine in alkaline ferrieyanide.¹⁵⁶ An intermediate red-colored complex forms between 4-aminoantipyrine and ferrieyanide, which decomposes. An alternative is to isolate by cation-exchange resin and couple with *p*-nitroaniline. If catecholamines are present, one uses Millon's reagent.¹⁵⁷

Yet another method is to oxidize the phenylephrine to *m*-hydroxybenzaldehyde with iodate.¹⁵⁸ This is read in the ultraviolet. There is no interference by α -(2-pyrimidylamino) methyl benzyl alcohol or by 2-(β -hydroxyphenethylamino)-sym-triazine, as they are not oxidized. Ephedrine, phenylpropylamine, and phenylamidol give the same reaction.

¹⁵⁵ J. Newsome and D. L. H. Robinson, *Trans. Roy. Soc. Trop. Med. Hyg.* **54**, 454-8 (1960).

¹⁵⁶ C. F. Hiskey and N. Levin, *J. Pharm. Sci.* **50**, 393-5 (1961); K. T. Koshy and H. Mitchner, *J. Pharm. Sci.* **52**, 802-3 (1963).

¹⁵⁷ Konosuke Murai, *Yakugaku Zasshi* **80**, 544-7 (1960).

¹⁵⁸ Lester Chafetz, *J. Pharm. Sci.* **52**, 1193-5 (1963).

Phenylephrine forms a chelate with copper, similar to that formed by ephedrine. Follow the technic for ephedrine, page 115, but read at 580 $m\mu$.

To determine phenylephrine in the presence of tetracaine, the solution is read at 241 $m\mu$ at pH 8 and pH 12.¹⁵⁹ The difference is $E_{1cm.}^{1\%} = 241$. To determine phenylephrine in the presence of procaine, extract most of the latter with chloroform from an alkaline solution. Then read at 237 $m\mu$ at pH 8 and 12. The difference is $E_{1cm.}^{1\%} = 410$.

Procedure—*By 4-aminoantipyrine*. Mix a 3-ml. sample containing 0.15-0.45 mg. of phenylephrine hydrochloride with 1 ml. of 4% potassium ferricyanide solution. Add about 44 ml. of 2% sodium tetraborate solution and 1 ml. of 3% 4-aminoantipyrine solution. Dilute to 50 ml. with the tetraborate solution and mix well. Read the absorption at 490 $m\mu$ at once against a reagent blank.

Alternatively, mix the 3 ml. of sample, 1 ml. of ferricyanide solution, 15 ml. of 0.05 *M* tris(hydroxymethyl)aminomethane buffer for pH 9, 15 ml. of isopropanol, and 1 ml. of the 3% 4-aminoantipyrine reagent. Dilute to 50 ml. with the buffer and mix. Read after 30 minutes at 490 $m\mu$ against a reagent blank. Common tablet ingredients may cause a cloudy solution by this technic when the isopropanol is added. They do not so interfere with the first technic.

By iodate. Shake 5 ml. of sample containing about 2.25% of phenylephrine hydrochloride with 1 ml. of saturated solution of sodium bicarbonate and 0.5 ml. of 2% sodium iodate solution for 10 minutes. Add 1-2 drops of concentrated hydrochloric acid. Extract with 5 successive 5-ml. portions of chloroform. Dilute the combined chloroform extracts to 25 ml. with the same solvent. Extract a 15-ml. portion with 10 ml. of 0.2% sodium hydroxide solution. Filter the alkaline extract and read it at 237 $m\mu$.

AMINOMETHYLCYCLOHEXANECARBOXYLIC ACID

This test substance is separated from deproteinized blood by column chromatography and read as the picrate.¹⁶⁰

Procedure—Mix 1 ml. of blood with 5 ml. of 1% picric acid solution. Centrifuge at 3000 rpm for 30 minutes at 4°. Pass the centrifugate

¹⁵⁹ B. Salvesen, *Medd. Norsk. Farm. Selsk.* **24**, 185-97 (1962).

¹⁶⁰ Yumiko Takada, Akikazu Takada and Utako Okamoto, *Keio J. Med.* **13**, 115-21 (1964).

through a 2×5 -cm column of 200-400 mesh Dowex 2-X8. Concentrate the percolate to 3 ml. at 40° . Adjust the pH to 2 and pass through a 9×15 -cm. column of Amberlite IR-120. Wash the column with 0.38 *N* citrate buffer at pH 4.26, collecting the eluate in 10 ml. fractions. The aminomethylcyclohexanecarboxylic acid appears in fractions 25 to 35. Read it at 570 $m\mu$.

ϵ -AMINOCAPROIC ACID

Follow the method for aminomethylcyclohexanecarboxylic acid, but make the last column of Amberlite 0.9×30 cm.

EPIRENAMINE

Epirenamine in pharmaceutical samples is measured by the indophenol reaction.¹⁶¹

Procedure—Dilute a sample in 1:110 hydrochloric acid solution with water, so that the final solution contains 0.01-0.1 mg. per ml. Mix a 2-ml. aliquot with 2 ml. of Sorensen buffer at pH (cf. Vol. 1, pp. 175-6). Add 0.3 ml. of 0.05% *p*-aminodiphenylamine solution, 3 ml. of isobutanol, and 0.7 ml. of 0.01% sodium hypochlorite solution. Keep for 5 minutes at $5-30^\circ$. To the isobutanol layer, add 0.2 ml. of absolute ethanol and read at 625 $m\mu$.

DIMETHYLAMINE

For its determination by pyrocatechol, see dibutylamine, page 137.

DIETHYLAMINE

For its determination by pyrocatechol, see dibutylamine, page 137.

DIBUTYLAMINE

Dibutylamine is determined by cobaltous chloride and *o*-dianisidine.¹⁶² See 1,2-*bis*(methylthio)ethane, Volume IIIA, page 454, for discussion.

¹⁶¹ Konosuke Murai, *Yakagaku Zasshi* **81**, 330-4 (1961).

¹⁶² Jacob S. Harker, Irwin Master, Louis E. Mattison and Benjamin Witten. *Anal. Chem.* **29**, 82-4 (1957).

A color reaction in acetone with pyrocatechol is quantitatively applicable not only to dibutylamine, but also to dimethylamine, diethylamine, diethanolamine, piperidine, pyrrolidine, ephedrine, *N*-methylaniline, L-proline, and pyrrolidone.¹⁶³ The amounts of reagents are the same, including the addition of acetone. Primary amines interfere; tertiary amines do not.

Procedure—*By cobaltous chloride and dianisidine.* As a reagent, dissolve 0.2 gram of cobaltous chloride hexahydrate in 5 ml. of absolute ethanol and dilute to 100 ml. with anhydrous ether. Add 2 drops of 30% hydrogen peroxide. To a 1-ml. sample containing up to 0.4 mg. of dibutylamine in ethyl ether, add 1 ml. of reagent. Heat at 60-80° until the solvent has evaporated, and cool. Add 0.2 ml. of 0.04% *o*-dianisidine solution in absolute ethanol and 4 ml. of a buffer for pH 1.5. Read immediately at 448 m μ .

By pyrocatechol. To a sample containing 0.022-0.06 mg. of dibutylamine in 0.5 ml. of acetone, add 1 ml. of 0.1% catechol solution and 2 mg. of silver oxide. After 10 minutes at room temperature, add 2 ml. of acetone and read at 510 m μ .

DIETHANOLAMINE

For determination by pyrocatechol, see dibutylamine above.

DI-ISOPROPYLAMINE-DICHLOROACETATE

Di-isopropylamine-dichloroacetate is nitrosated in 50% acetic acid at 40-60°.¹⁶⁴ After making the solution alkaline, the *N*-nitrosodiisopropylamine is extracted for reading.

Procedure—Mix 2 ml. of neutral aqueous sample with an equal volume of glacial acetic acid. Add 2 ml. of 10% sodium nitrite solution and let it stand for 5 minutes. Add sufficient 20% potassium hydroxide solution to make the solution definitely alkaline, and extract with 5 ml. of cyclohexane. Read the extract at 235 m μ .

¹⁶³ Jaroslav Bartos, *Ann. Pharm. Franc* 20, 478-9 (1962).

¹⁶⁴ Masao Maruyama and Kazuo Hasegawa, *Japan Analyst* 10, 518-22 (1961).

ZECTRAN, 4-DIMETHYLAMINO-3,5-XYL METHYLCARBAMATE

Zectran, after hydrolysis to the corresponding phenol, *4-dimethylamino-3,5-xyleneol*, is determined in alkaline solution by luteoarsenotungstic acid reagent.¹⁶⁵ Due to the sensitivity of this Folin-Ciocaltin reagent to reducing agents, an extensive cleanup of the sample solution is essential. The reagent does not react with Zectran. Therefore it can be used for determination of the free phenol before hydrolysis of Zectran. Recoveries of 0.1-2.5 ppm. from peaches are in the 80-95% range for each.

Procedure—As luteoarsenotungstic acid reagent, add 200 ml. of water to 50 grams of arsenic pentoxide and 20 grams of sodium tungstate dihydrate. Reflux for 1.5 hours. Add 50 grams of lithium sulfate monohydrate and 5 or 6 drops of bromine. Boil for 8 to 10 minutes to drive off the excess bromine. Cool, dilute to 250 ml. with water, and filter. Store in amber glass-stoppered bottles.

Carbamate and phenol. Peaches. Grind up the sample and weigh a 100-gram portion. Add it to a 500-ml. French-square bottle with 10 grams of Hyflo Supercel and 350 ml. of methanol. Roll mechanically for an hour. Filter on a Büchner funnel and press the solids to squeeze out the methanol. Wash the bottle and solids with 25, 25, and 25 ml. of methanol. Dilute the combined extracts to 500 ml.

Mix 200 ml. of sample solution and 500 ml. of water in a separatory funnel and add 10 ml. of a saturated solution of sodium bicarbonate. Extract with 100, 50, and 50 ml. of benzene. Combine these extracts and extract with 25 and 25 ml. of 1:17 sulfuric acid. Wash the combined acid extracts with 25, 25, and 25 ml. of ether. Wash a 50-ml. portion of the sample in sulfuric acid with 25, 25, and 25 ml. portions of chloroform.

Add 50 ml. of 14% potassium hydroxide solution and mix. After about 3 minutes, add 20 ml. of 1:17 sulfuric acid. Cool with cold water. Cautiously add 25 ml. of saturated sodium bicarbonate solution with shaking to eliminate carbon dioxide.

Extract with 25 and 25 ml. of thiophene-free benzene. Wash the combined extracts with 10 ml. and 10 ml. of water. Extract the benzene solution with 5 ml. and 5 ml. of 1:68 sulfuric acid. Add 1 ml. of reagent to the acid extract. Add 4 ml. of 15% sodium bicarbonate solution in 0.5

¹⁶⁵ R. P. Marquardt and E. N. Luce, *J. Agric. Food Chem.* **11**, 418-22 (1963).

ml. increments, shaking out the carbon dioxide after each addition. Then add 6 ml. more. Dilute to 25 ml. and mix well. After 5-15 minutes, read against water at 700 m μ .

The phenol. Proceed as for carbamate and phenol, starting with "Add 50 ml. of 14% potassium . . .," but omit the hydrolysis of the carbamate. Instead, neutralize the washed sample by cautious addition of 25 ml. of saturated sodium bicarbonate solution. Shake to eliminate as much of the dissolved carbon dioxide as possible. Continue from "Extract with 25 ml. and 25 ml. of thiophene-free benzene."

The carbamate. Subtract a determination of the phenol alone from one of carbamate and phenol.

Undelinted cottonseed. The xylenol. Grind the sample in a Wiley mill. Weigh a 100-gram sample into a 1-liter French-square bottle. As benzene reagent, mix 85% of thiophene-free benzene with 15% of technical-grade toluene. Add 550 ml. of this and 20 grams of Hyflo Supercel. Roll the bottle mechanically for 30 minutes. Filter on a Büchner funnel.

Return the cottonseed to the bottle, leaving most of the filter aid on the funnel. Add 500 ml. of the benzene mixture and roll for 30 minutes. Filter on the same funnel and dilute the combined filtrates to 1 liter with the benzene mixture. Extract the xylenol from a 400-ml. aliquot with 25 and 25 ml. of 1:17 sulfuric acid. Wash the combined extracts with 25, 25, and 25 ml. of ether.

Proceed as for the carbamate and phenol in peaches, from "Cautiously add 25 ml. of saturated sodium bicarbonate . . ."

4-DIMETHYLAMINO-3,5-XYLENOL

See Zettran, page 138.

METHADONE, DL-6-DIMETHYLAMINO-4,4'-DIPHENYL-3-HEPTANONE

Methadone may be read in the ultraviolet after aqueous extraction.¹⁶⁶ However, extraction with chloroform and steam distillation is often required.¹⁶⁷ It may also be read in the infrared after chloroform extraction (cf. Vol. IV, p. 39). Extraction with chloroform can be used to separate

¹⁶⁶ H. J. van der Pol and R. F. Rekker, *Pharm. Weekblad* **96**, 41-S (1961).

¹⁶⁷ M. J. Pro, *J. Assoc. Agr. Chemists* **42**, 177-80 (1959).

methadone hydrochloride from codeine sulfate, morphine hydrochloride, morphine sulfate, morphine tartrate, sodium phenobarbital, or sparteine sulfate. Methadone hydrochloride may be separated from the following compounds by steam distillation: acetylsalicylic acid, caffeine, cocaine hydrochloride, codeine phosphate, dihydromorphinone hydrochloride, Dromoran, mannitol, milk sugar, narcein alkaloid, orthoform, papaverine hydrochloride, quinidine, quinine hydrochloride, starch, talc, and thebaine alkaloid. Methadone is also nitrated and the sodium salt read at 420 $m\mu$.¹⁶⁸

Procedure—*In the ultraviolet. Tablets.* Prepare an aqueous slurry of Filter-Cel. With the aid of suction, pack a $\frac{3}{8}$ inch layer in a fritted glass funnel of 20 mm. diameter in a filtration apparatus. Dissolve a sufficient number of tablets to contain 20-40 mg. of methadone hydrochloride in 10 ml. of water. Allow the insoluble material to settle and decant into the fritted funnel. Wash the residue with three 10-ml portions of water and pour each through the filter. Dilute to 50 ml. with water and read at 292.5 $m\mu$ against water.

Powdered samples if contaminated. Add a 100-mg. sample to 10 ml. of water. Filter and pass through Filter-Cel as previously described. Evaporate the filtrate to 10 ml., cool, and transfer to a separatory funnel. Wash the beaker with 20 ml. of chloroform and add to the funnel. Shake for 2 minutes. Repeat the extraction 3 times and evaporate the chloroform to dryness at 100°. Dissolve in 10 ml. of warm 1:110 hydrochloric acid and transfer to the steam distillation apparatus. Wash the beaker with two 10-ml. portions of 5% disodium hydrogen phosphate solution at pH 8.7-9.5 and add each wash to the apparatus. Steam distil and collect 200 ml. of distillate in a flask containing 10 ml. of 1:110 hydrochloric acid. Wash down the sides with 30 ml. of ethanol and dilute to 250 ml. with water. Prepare a reference solution containing 10 ml. of 1:110 hydrochloric acid, 30 ml. of 95% ethanol, and 210 ml. of water. Read the sample against the reference at 292.5 $m\mu$.

In the infrared. Add 10 ml. of water to a number of tablets containing 30 mg. of methadone hydrochloride. Add 20 ml. of chloroform and shake for 2 minutes. Filter the chloroform layer through a cotton plug. Repeat the extraction with three 20-ml. portions of chloroform and slowly evaporate the chloroform to dryness at 100°. Cool, add 10 ml. of chloro-

¹⁶⁸ M. Skóra, *Dissert. Pharm.*, Krakow 15, 433-7 (1963).

form, swirl, and read at $5.86\ \mu$ relative to chloroform. Determine the absorbance of a standard solution containing 3 mg. of methadone hydrochloride per ml. in chloroform relative to chloroform. Draw the baseline between the minima at $5.75\ \mu$ and $6.00\ \mu$.

As the nitrate. Tablets. Dissolve a powdered sample containing about 10 mg. of methadone in water. Filter, and wash the filter with water containing 2 drops of 1:10 hydrochloric acid. Dilute the filtrate to 10 ml. Make 1 ml. alkaline with 10% sodium carbonate solution and filter the precipitated methadone base. Dissolve in ether, dry the ether solution with anhydrous sodium sulfate, and evaporate the ether.

To the residue containing about 1 mg. of methadone, add 2 ml. of a 1:1 mixture of nitric acid of specific gravity 1.5 and concentrated sulfuric acid. Heat at 100° for 30 minutes, and cool. Add 5 ml. of water followed by 20% sodium hydroxide solution to adjust to pH 10. Add 3 drops of 5% gelatine solution and dilute to 25 ml. Read at $420\ m\mu$.

TERTIARY ALIPHATIC AMINES

Tertiary aliphatic amines may be determined by reaction with aconitic anhydride or by reaction with chloranil.¹⁶⁹ When used together, the methods can be used for quantitatively differentiating between tertiary amines and amine salts or quaternary amines. With aconitic anhydride, the sensitivity is 0.003 mg. per ml.; with chloranil, 0.05 mg. per ml.

Straight-chain tertiary aliphatic amines from trimethyl through triamylamine, including 2-diethylaminoethanol and *N*-methyl diisopropylamine show the same order of sensitivity. Triethanolamine shows poor sensitivity with both methods. Triisopropanolamine shows a sensitivity of 25% of that of tributylamine with aconitic anhydride and produces no color with chloranil. A sensitivity for tetramethylphosphonium chloride is 0.01 mg. per ml. Amides do not react and tertiary aromatic amines show poor sensitivity.

Interferences from Lewis bases, such as sodium acetate and sodium phosphate, present from impurities from solvents, reagents, or glassware in the cis-aconitic anhydride system is avoided by using borosilicate glass burets and pipets and purifying the reagents. Beer's law is followed.

A micro method for cholines by iodine, on page 200, is applicable to tertiary amines.

¹⁶⁹ Samuel S. Sass, Joyce J. Kaufman, Arturo A. Cardenas and John J. Martin, *Anal. Chem.* **30**, 529-31 (1958).

Procedure—*By chloranil*. Wash toluene with hydrochloric acid, sulfuric acid, and water to remove pyrrole and thiophene. Distil the washed material from calcium chloride. Dissolve a sample in toluene so that a 3-ml. aliquot contains 0.1-0.8 mg. of tertiary amine. Add 1 ml. of 1% chloranil solution in toluene and heat at 100° for 15 minutes. Cool for 5 minutes and read the green color at 610 m μ .

By aconitic anhydride. Dissolve 0.25 gram of aconitic anhydride in 40 ml. of acetic anhydride and dilute to 100 ml. with toluene. Age for 24 hours. Prepare toluene as described under the chloranil method. If the sample is relatively insoluble, mix in advance with acetic anhydride and dilute with toluene to a 1:1 ratio. With tetraalkylphosphonium salts, dissolve the sample in acetic anhydride and dilute with toluene to a 1:1 ratio. Otherwise, dissolve a sample in toluene so that a 2-ml. aliquot contains 0.02-0.07 mg. of tertiary amine or amine salt. Add 1 ml. of aconitic anhydride reagent, heat for 15 seconds at 100°, and let stand for 15 minutes. Add 5 ml. of toluene and read after 15 minutes at 500 m μ .

TRIMETHYLAMINE

The yellow color of trimethylamine picrate in toluene (Vol. IV, p. 40) is read as a measure of the deterioration of fish.¹⁷⁰

Sample—*Fish*. Grind 10 grams in a mortar. Add 10 ml. of 20% trichloroacetic acid solution and grind to a homogeneous paste. Allow to settle. If turbidity interferes, adjust to neutrality, clarify by addition of mercuric acetate solution, and filter.¹⁷¹ The interfering substance is probably cadaverine. To 5 ml. of the supernatant liquid, add sequentially 1.5 ml. of 38% formaldehyde, 10 ml. of toluene, and 15 ml. of a saturated potassium carbonate solution, mixing after addition of each reagent. Allow the emulsion to break and add 5 ml. of the toluene layer to a small amount of anhydrous sodium sulfate to dry it. Pour the toluene into a tube containing 5 ml. of 0.1% pieric acid solution in trichloroethylene and read at 410 m μ .

¹⁷⁰ A. Fernandez del Riego and A. Rodriguez de las Heras, *bol. inst. espanol. oceanog.* **65**, 1-19 (1954).

¹⁷¹ Kaname Saito and Muneo Sameshima, *Nippon Nôgei-Kagaku Kaishi* **30**, 531-7 (1956).

TRIISOOCTYL AMINE

Up to 5 mg. of triisooctyl amine may be determined by complex formation with cobalt thiocyanate.¹⁷² The complex is developed in dilute sulfuric acid and is extracted with carbon tetrachloride. Sodium dihydrogen orthophosphate suppresses interference from iron. Uranium does not interfere.

Procedure—To prepare the cobalt-thiocyanate reagent, dissolve 10 grams of cobalt nitrate and 10 grams of sodium thiocyanate in 100 ml. of water. The sample should be in 2.5% sulfuric acid solution. To a solution containing up to 5 mg. of triisooctyl amine, add sufficient sodium dihydrogen orthophosphate to complex any ferric iron present. Shake vigorously. In solutions containing 0.5% of iron, 5-10 grams of sodium dihydrogen orthophosphate is necessary. Add 20 ml. of cobalt-thiocyanate reagent, shake for 1 minute, and let stand for 2 minutes. Extract the complex by shaking with 10 ml. of carbon tetrachloride for 1 minute. Filter the organic layer and read at 620 $m\mu$ against carbon tetrachloride.

2'-(β -DIETHYLAMINOETHOXY)-3-PHENYLPROPIOPHENONE

A reagent for 2'-(β -diethylaminoethoxy)-3-phenylpropiophenone hydrochloride is ammonium reineckate.¹⁷³ Beer's law is followed at 395 $m\mu$ for 0.01-0.05 mg. per 20 ml. Ammonium reineckate may be replaced by phosphomolybdic acid. The solution is then read at 400 and 450 $m\mu$.

Procedure—*Pharmaceutical tablets.* Heat a 20-ml. aqueous sample containing 0.01-0.05 mg. of 2'-(β -diethylaminoethoxy)-3-phenylpropiophenone hydrochloride at 100° for 5 minutes. Acidify with hydrochloric acid, using Congo red indicator. Add dropwise 6 ml. of a saturated ammonium reineckate solution. After 1 hour, filter the precipitate. Wash with 1:24 hydrochloric acid and dissolve the precipitate in acetone. Dilute to 25 ml. with acetone and read at 395 $m\mu$.

SYMPATHOMIMETIC AMINES

The sympathomimetic amines, tyramine, mescaline, norsympathol, and norveritol are developed by *p*-nitroaniline and nitrous acid.¹⁷⁴ Beer's

¹⁷² Allan W. Ashbrook, *Analyst* **84**, 177-9 (1959).

¹⁷³ J. Giusti and G. F. DiPaco, *Boll. chim. farm.* **100**, 425-9 (1961).

¹⁷⁴ G. Vistoli, *Boll. chim. farm.* **94**, 133-7 (1955).

law is followed at 520 $m\mu$ for 0.5 mg. of *tyramine*, at 555 $m\mu$ for 0.4 mg. of *mescaline*, at 580 $m\mu$ for 0.3 mg. of *norsympathol*, and at 584 $m\mu$ for 0.5 mg. of *norveritol* per ml.

This class of compounds can be read at 253.7 $m\mu$.¹⁷⁵ They include the phenylalkylamines and phenylalkanolamines such as benzedrine, Pervitin, ephedrine, norephedrine, and Preludin; phenolic bases such as phenolalkylamines, tyramine, and veritol; *p*-phenylalkanolamines such as Sympatol, Suprifen, Vasculat, and Dilatol; *m*-phenylalkanolamines such as Adrianol and Effortil; 3,4-diphenylalkanolamines such as Aludrin, adrenaline, noradrenaline, and dihydroxyephedrine; and diphenylketo bases such as adrenalone and Asthma-Tropon.

The sympathomimetic amines that react by oxidation by iodate, and reading the arylaldehyde form in the ultraviolet, include ephedrine, phenylephrine, phenylpropanolamine, and phenylramidol.¹⁷⁶

Procedure—To prepare the reagent, dilute 0.69 gram of *p*-nitroaniline and 10 ml. of concentrated hydrochloric acid to 100 ml. with water. At 0°, mix 5 ml. with 1 ml. of hydrochloric acid and 3 ml. of 0.7% sodium nitrite solution. After 6 minutes, dilute to 100 ml. with water.

To a 2-ml. sample containing a sympathomimetic amine, add 5 ml. of reagent and let stand for 1 hour. Add 5 ml. of 1.1% sodium carbonate solution. After 15 minutes, add 1 ml. of 10% sodium hydroxide solution. After 10 minutes, dilute to 50 ml. with water. Extract a 25-ml. aliquot with 15 ml. of butanol. Centrifuge the butanol extract and read at 520 $m\mu$ after 4 hours for tyramine, at 555 $m\mu$ for mescaline, at 580 $m\mu$ for norsympathol, and at 584 $m\mu$ for norveritol.

HISTAMINE, β -IMIDAZOLYL-4-ETHYLAMINE

Histamine is extracted into *n*-butanol from alkalized perchloric acid tissue extracts, returned to aqueous solution, and condensed with *o*-phthalaldehyde to yield a highly fluorescent product^{177,178,179} (cf. Vol. III, pp. 43-7). Histamine and serotonin may be determined in the same sample.¹⁸⁰

¹⁷⁵ H. Thies and Z. Ozbilici, *Arch. Pharm., Berlin* **295**, 194-6, 715-18 (1962).

¹⁷⁶ Lester Chafetz, *J. Pharm. Sci.* **52**, 1193-5 (1963).

¹⁷⁷ Parkhurst A. Shore, Alan Burkhalter and Victor H. Cohn, Jr., *J. Pharmacol. Exper. Therapy* **127**, 182-6 (1959).

¹⁷⁸ J. A. Oates, E. Marsh and A. Sjoerdsma, *Clin. Chim. Acta* **7**, 488-97 (1962).

¹⁷⁹ H. Zachariae, *J. Lab. Clin. Invest.* **15**, 173-8 (1963).

¹⁸⁰ Michael Cais, *Med. Exptl.* **5**, 73-6 (1961).

In fluorescent determination of histamine with *o*-phthalaldehyde, tissue samples may be homogenized with trichloroacetic acid to liberate histamine and passed through an anion exchange resin to remove histidine, amino acids, and organic anions.¹⁸¹ The effluent from the column should not exceed pH 11.4 to prevent interference from amines. The salt concentration of the sample should not exceed 0.005 *M*, since higher salt concentrations produce higher pH and low histamine values. An alternative that has been applied to micro samples is to sorb on a column of cellulose acid succinate and elute with 1:110 hydrochloric acid in 50% ethanol.¹⁸² The following do not produce significant fluorescence at 450 m μ : acetylhistamine, 1-methyl-4-(β -aminoethyl)imidazole (1,4-methylhistamine), *N*-alkyl histamines with *n*-propyl and dimethyl side chain, imidazol, imidazolacetic acid, 1,5-methylhistidine, 1,4-methylimidazolacetic acid, urocanic acid, carnosine, anserine, serotonin, 3,4-dihydroxyphenylethylamine, norepinephrine, spermine and spermidine.

Histidine and histidylhistidine produce fluorophores with the reagent, which are not extracted with butanol and therefore do not interfere. Ammonia, in concentrations greater than 0.004 mg. per ml., produces fluorescence but does not interfere, since it is not extracted with butanol. However, concentrations such as 0.15 mg. of ammonia in urine samples does interfere. Beer's law is followed for 0.00005-0.0005 mg. per ml.

By modification of the amounts of sample and reagents used, microquantities of histamine may be determined after condensation with *o*-phthalaldehyde.¹⁸³ In the presence of histidine, samples should be passed through Decalso.

The diazotized *p*-nitroaniline method for histamine is modified to stabilize the color.¹⁸⁴ The most uniform color development is achieved by addition of the diazonium salt when the sample solution is at pH 8.3-8.6.¹⁸⁵ This pH is established by addition of 1 ml. of a coupling buffer of sodium metaborate and sodium carbonate at pH 10.9 to the acidified purified histamine. With this adaptation, it is not necessary to saturate the solution with borax buffer.

Histamine is purified by the formation of a Schiff base with benzalde-

¹⁸¹ Leon T. Kremzner and Irwin B. Wilson, *Biochim. et Biophys. Acta* **50**, 364-7 (1961).

¹⁸² J. W. Noah and A. Brand, *J. Lab. Clin. Med.* **62**, 560-10 (1963).

¹⁸³ Joseph W. Noah and Alta Brand, *J. Allergy* **32**, 236-40 (1961).

¹⁸⁴ O. S. Sager and William Horwitz, *J. Assoc. Offic. Agr. Chemists* **40**, 892-904 (1957).

¹⁸⁵ David W. Williams, *ibid.* **43**, 431-3 (1960).

hyde. The base is decomposed by cotton acid succinate and the histamine is adsorbed on the carboxyl groups of the adsorbent. Histamine is then displaced with sulfuric acid, which also regenerates the cotton adsorbent. The extraction of the red azo dye with methyl isobutyl ketone eliminates impurities and stabilizes the color. Washing with barbital buffer at pH 7.7 converts extracted red compounds to yellow. Beer's law is followed up to 0.025 mg. Glassware for this method should be cleaned with chromic acid cleaning solution, not with soap.

The complex formed by the reaction of histamine with 2,4-dinitrofluorobenzene is concentrated in methyl *n*-hexyl ketone and hydrochloric acid.¹⁸⁶ The samples are run through a column of Decalso, which is heated to increase the amount of histamine eluted. A combination of this procedure with the ketone extraction eliminates interference from most amines, ergothionine, glutathionine, adenine, guanine, cytosine, adenosine, imidazole, and dinitrophenol from decomposed reagent. Spermine, spermidine, putrescine, cadaverine, histidinol, and agmatine interfere.

Diazotized *p*-bromoaniline is used to develop an orange-red color with histamine.^{187,188} This is extractable with butanol. Another azo dye is formed with *p*-nitrobenzene diazonium chloride.¹⁸⁹ With this reagent, histamine is chromatographed in Amberlite IRC-50 at pH 4.6, eluted with hydrochloric acid, and read at 550 m μ . Histidine and tyrosine are removed from the column with acetate buffer at pH 4.6, and lipid deposits are washed off with acetone.

In the determination of histamine with *p*-sulfonic acid, diazonium salt, diethylenetriaminepentaacetic acid is used to couple the following interfering ions: cobaltous, cuprous, cupric, nickel and ferric.¹⁹⁰ A mixture of histamine and histidine gives the former by difference. The sample is passed through a column of Amberlite IR-100. The *p*-sulfonic acid diazonium chloride development is applied before and after such treatment.¹⁹¹ Reagents for histidine are applicable in some cases for the

¹⁸⁶ Oliver H. Lowry, Helen Tredway Graham, Frances B. Harris, Martha K. Priebe, Ansel R. Marks and Robert U. Bregman, *J. Pharm. Exptl. Therapy* **112**, 116-126 (1964); cf. Jun Ishihara, *Nippon Saikigaku Zasshi* **12**, 183-8 (1957).

¹⁸⁷ Thomas D. Lyons and A. C. Andrews, *Trans. Kanson Acad. Sci.* **58**, 935-8 (1955).

¹⁸⁸ Jacques Baraud and Louis Genevois, *Bull. soc. chim. France* **1956**, 681-3.

¹⁸⁹ Atsushi Tsuda and Tetsuo Tomiyama, *Nippon Saisan Gakkaishi* **25**, 451-6 (1959).

¹⁹⁰ A. C. Andrews and T. D. Lyons, *Anal. Chem.* **29**, 1325-7 (1957).

¹⁹¹ S. K. Ganguly and Hrishikesh Bhattacharya, *Indian J. Pharm.* **16**, 72-4 (1954).

determination of histamine. Among these are *p*-nitrobenzoyl chloride,¹⁹² and pyridine-and-iodine.¹⁹³ (See Vol. IV, p. 183, and this volume, p. 269).

Sample—Tissue. Add sufficient sodium chloride to 0.4% sodium hydroxide solution so that an excess of solid remains. Homogenize the sample in 9 volumes of 5% perchloric acid solution. After 10 minutes, centrifuge. Mix 0.5 ml. of 20% sodium hydroxide solution, 1.5 gram of solid sodium chloride, and 10 ml. of *n*-butanol. Add a 4-ml. aliquot of the supernatant sample liquid. Shake for 5 minutes to extract histamine and centrifuge. Aspirate the aqueous phase. Shake the butanol phase with 5 ml. of salt-saturated 0.4% sodium hydroxide solution for 1 minute to remove any residual histidine. Centrifuge, and add an 8-ml. portion of the butanol to a mixture of 2.5-4.5 ml. of 0.4% sodium hydroxide solution and 15 ml. of heptane. Shake for 1 minute. Centrifuge, and develop histamine in the aqueous phase with *o*-phthalaldehyde by the first technic under the procedure.

Tissue. For determining histamine and serotonin. Homogenize a 2-3 gram sample in 10 ml. of 4% perchloric acid. Add 10 ml. of 4% perchloric acid and adjust the pH to 11 with 2 grams of anhydrous sodium carbonate. Saturate the suspension with 6 grams of sodium chloride and shake for 1 hour with 25 ml. of water-saturated butanol. Centrifuge, remove the butanol layer, and wash it twice with 5 ml. of boric acid buffer at pH 10. Shake for 30 minutes with 4 ml. of 1:110 hydrochloric acid and 20 ml. of 40-70° petroleum ether. Develop an aliquot of the aqueous layer for histamine by *o*-phthalaldehyde. Develop another aqueous aliquot for serotonin by fluorescence, page 157.

Tissue. Purification by anion-exchange resin. Homogenize a 1-gram sample with 4 ml. of 5% trichloroacetic acid and centrifuge. Add 1 ml. of the supernatant liquid to a mixture of 0.5 ml. of 2% sodium hydroxide solution and 5 ml. of 0.01 *M* phosphate buffer. Dilute to 10 ml. with water. To prepare the resin, convert 50-100 mesh Dowex-1 X 8, Bio Rad-1 X 8 or IRA-410 to the acetate-hydroxide form in a large column with several volumes of 10.2% sodium acetate trihydrate-0.2% sodium hydroxide. Wash the resin with water and store in a refrigerator. As a column, use a 10-ml. pipette with the upper constriction removed. Fit with a glass wool plug. Add the resin slurry. Allow to settle and adjust the resin

¹⁹² Robert W. Cowgill, *Anal. Chem.* **27**, 1521-3 (1955).

¹⁹³ P. M. Newman and J. H. Turnbull, *Biochem. J.* **74**, 379-82 (1960).

volume to 6 ml. Before adding the sample, lower the water to the resin surface. Add 5 ml. of the sample solution to the anion-exchange column. Adjust the flow rate to 1 ml. per minute with a screw clamp. When the sample reaches the top surface of the resin, add 0.05-0.01 *M* phosphate buffer as a wash.

Discard the first 1-2 ml. of effluent and collect 10 ml. for determination by *o*-phthalaldehyde using the second technic under the procedure.

*Fish.*¹⁹⁴ Extract a 10-gram sample with chloroform and adjust the extract to pH 4.6. As an 8 × 55-mm. chromatographic column, use Amberlite CE-50, Type 1, 100-200 mesh adjusted to pH 4.6 with 2 *N* acetate buffer. Pass an aliquot of the chloroform solution through the column and follow with 80 ml. of the buffer for pH 4.6. Elute the histamine with 8 ml. of 1:55 hydrochloric acid. This and any necessary washings constitute the sample.

Histidine decarboxylase and other samples where histamine is not bound. Add a 2-ml. sample containing not more than 0.05 *M* salt and containing 0.00001-0.0005 mg. of histamine per ml. to a 3-ml. column of anion-exchange resin. Discard the first ml. of effluent and collect 4.5 ml. of effluent in a flask containing 0.5 ml. of 30% trichloroacetic acid solution. Centrifuge. Develop by the second technic for *o*-phthalaldehyde but use 0.4 ml. of 6% sodium hydroxide solution and 0.2 ml. of 25% phosphoric acid.

Blood. Macro. Hemolyze 5 ml. of oxalated blood with 5 ml. of water and 0.5 ml. of 70% perchloric acid. Shake for 10 minutes and follow the first technic by *o*-phthalaldehyde.

Micro. Centrifuge a 2-ml. sample at 3500 rpm. for 30 minutes. To 1 ml. of plasma, add 0.112 ml. of 50% trichloroacetic acid solution. Mix. and let stand for 30 minutes, mixing at 15 minute intervals. Centrifuge at 3500 rpm. for 40 minutes to remove the protein precipitate. Develop 0.5 ml. of the supernatant liquid with *o*-phthalaldehyde, micro method.

Plasma. Prepare an adsorption column and hot box as illustrated in Figure 4. The narrow section at the bottom of the column contains the adsorbent and is placed in the hot box. With the aid of flowing water, add a few mm. of 40-60 mesh acid-washed sand to the column. Add

¹⁹⁴ Toshiharu Kawabata, Yutaka Uchida, and Taeko Akamo, *Nippon Saisei Gak-kai* 26, 1183-91 (1960).

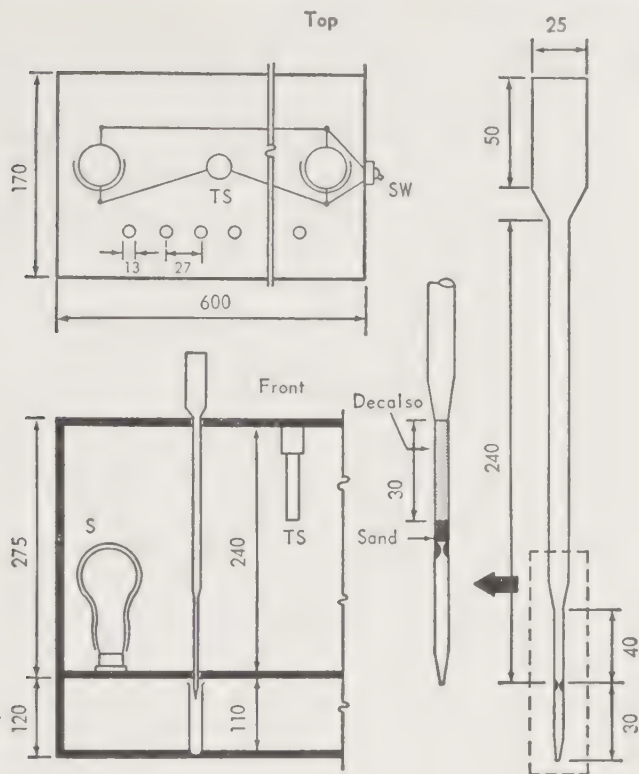


FIG. 4.

Adsorption column and hot box. Numbers give dimensions in millimeters. Inner diameter middle segment of column, 8 mm.; Decalso segment, 1.8 mm.; construction, 0.3 mm. TS = Fenwal thermal switch, SW = on-off switch, S = 100-watt lamp, partially surrounded by aluminum foil shield to prevent local overheating

enough Decalso, which has been boiled with 3% acetic acid, washed and dried, to make a 3-cm. column after packing. Adjust the column so that the flow rate is 0.1-0.5 ml. per minute.

Separate plasma quickly at a low temperature to avoid release of leukocyte histamine. Use 1.7% isotonic potassium oxalate solution as an anticoagulant. To 10 ml. of plasma, add 2 ml. of 20% trichloroacetic acid solution with vigorous mixing. After 30 minutes, centrifuge at high speed.

To 10 ml. of deproteinized plasma, add 0.1 volume of 32% sodium acetate solution to bring the pH to 4. Rinse with a little water into the Decalso column. After the sample flows through the column, wash with 5 ml. of water. Blow out the last drops of the water wash, place the column in the hot box, and add 0.5 ml. of 40% potassium bromide solution

just above the Decalso. Collect the eluate in 0.04 ml. of 0.014% sodium diethyldithiocarbamate solution. Develop with 2,4-dinitrofluorobenzene.

Whole blood. Treat 2 ml. with 2 ml. of 10% trichloroacetic acid solution and centrifuge. Follow the procedure for plasma, using 2 ml. of the whole blood extract, starting at "add 0.1 volume of 32% sodium acetate solution . . ."

*Urine.*¹⁹⁵ Adjust the pH of a sample to 7.5. Filter and mix with 0.15 M sodium phosphate buffer for pH 7.5. Pass this through a column of 100-200 mesh Amberlite IRC-50 and wash thoroughly with water. Elute the histamine from the washed column with 1:10 hydrochloric acid. Make the eluate alkaline by addition of 40% sodium hydroxide solution. Saturate with sodium chloride and extract the histamine with butanol. Wash the butanol extract with 0.4% sodium hydroxide solution saturated with sodium chloride and butanol. Dilute the butanol solution with heptane and extract with 1:110 hydrochloric acid. Develop by *o*-phthalaldehyde.

Canned tuna fish. To prepare the cotton acid succinate, dissolve 5 grams of anhydrous sodium acetate and 40 grams of succinic anhydride in 300 ml. of acetic acid. Cut 10 grams of absorbent cotton into strips and immerse in the solution. Attach a tube containing a drying agent and heat for 48 hours at 100°. Filter, wash with 9:1 hydrochloric acid followed by an alcohol wash. Dry in a vacuum oven at 100°.

Prepare the column by cutting or blowing out the bottom of a 15-ml. centrifuge tube. Wash a plug of cotton acid succinate of approximately 50 mg. with three 15-ml. portions of water and two 3-ml. portions of 95% ethanol. Allow the solvents to drip through the cotton and blow out the last portion of the solvent, using a 10-ml. syringe with the needle inserted through a rubber stopper. Pass the entire sample through a food chopper three times, mixing after each grinding. Blend the ground sample with 50 ml. of methanol in a high speed semi-micro container of a blender for 2 minutes. Rinse the lid and blender with methanol and add the rinsings to the sample. Heat at 60° for 15 minutes. Cool to 25°, and dilute to 100 ml. with methanol. Filter through folded paper. Dilute a 5-ml. portion of the filtrate to 100 ml. with water, disregarding any turbidity. To a 5-ml. aliquot, add 1 drop of chloride-free benzaldehyde and 0.2 ml. of 20% sodium hydroxide solution. The pH should be 12.4 to

¹⁹⁵ J. A. Oates, E. Marsh and A. Sjoerdsma, *Clin. Chim. Acta* 7, 488-97 (1962).

12.5. Shake vigorously 25 times. After 2 minutes, add 5 ml. of a 3:2 mixture of benzene and *n*-butanol to extract the Schiff base formed with histamine. Shake vigorously 25 times and allow 5 minutes for separation. Centrifuge if an emulsion forms. Transfer the upper layer to the cotton acid succinate tube. Re-extract the aqueous layer with 5 ml. of benzene and *n*-butanol solvent as before and add the upper layer to the cotton acid succinate column. Rinse the lip and sides of the column with 95% ethanol, blowing out excess solvent with a syringe as before. Wash the column with 3 ml. of 95% ethanol. Blow out, and wash with two 3-ml. portions of water which are also blown out. Discard the solvents and washings. Elute the histamine with 2 ml. of 1:89 sulfuric acid followed by two 2-ml. portions of water. Blow out after each of the additions. Cool the eluate for 5-10 minutes in ice. Develop with diazotized *p*-nitroaniline.

Procedure—By *o*-phthalaldehyde. *Macro*. To a 2-ml. extract of the sample containing 0.05-0.5 microgram of histamine, add 0.4 ml. of 4% sodium hydroxide solution and 0.1 ml. of 1% *o*-phthalaldehyde solution in absolute methanol. After 4 minutes, add 0.2 ml. of 1:3 hydrochloric acid, mixing thoroughly. Read the fluorescence within 30 minutes at 450 $m\mu$ with activation at 360 $m\mu$.

To correct for fluorescence in tissues or reagent, add all reagents to a separate aliquot of the tissue sample, reversing the order of addition of *o*-phthalaldehyde reagent and 1:3 hydrochloric acid. The addition of acid first prevents the condensation of reagent with histamine.

As a second technic, when purified by a resin column, to a 2-ml. portion, add 0.4 ml. of 2% sodium hydroxide solution and 0.1 ml. of 1% *o*-phthalaldehyde solution. After 3.5 minutes, add 0.2 ml. of phosphoric acid containing 144 grams per liter. Measure the fluorescence at 445 $m\mu$ with excitation at 345 $m\mu$.

Micro. To a 0.5-ml. sample containing 0.002-0.15 microgram of histamine, add 0.2 gram of sodium chloride and 0.06 ml. of 20% sodium hydroxide solution, mixing after each addition. Shake with 1.25 ml. of *n*-butanol. Allow the layers to separate and discard the aqueous portion. Wash the butanol with two 1-ml. portions of 0.4% sodium hydroxide solution saturated with sodium chloride. Add 1 ml. of the butanol layer to 1.5 ml. of *n*-heptane. Mix and allow the layers to separate. To 0.3 ml. of the acid layer, add 0.06 ml. of 4% sodium hydroxide solution. Mix and add 0.014 ml. of 0.1% *o*-phthalaldehyde solution. Let stand at room

temperature for 4 minutes. Add 0.025 ml. of 1:3 hydrochloric acid and read the fluorescence within 30 minutes.

By diazotized p-bromoaniline. As a stock solution, dissolve 0.9 gram of *p*-bromoaniline in 9 ml. of concentrated hydrochloric acid and dilute to 100 ml. with water. As the reagent, mix 1.5 ml. of this stock solution with 7.5 ml. of 5% sodium nitrite solution and dilute to 50 ml.

To an appropriate volume of sample, add 5 ml. of 1.2% sodium carbonate solution. Add 2 ml. of the reagent and mix. Extract the dye with 10 ml. of butanol and read at 500 $m\mu$.

By diazotized p-nitroaniline. To prepare the coupling buffer, dissolve 7.15 grams of sodium metaborate decahydrate and 5.7 grams of sodium carbonate in water and dilute to 100 ml. To prepare the barbital buffer, adjust a 1% sodium barbital solution to pH 7.7 with 1:15 acetic acid, using approximately 25-30 ml. of that acid. As a reagent, immerse 10 ml. of 0.1% *p*-nitroaniline solution in 1:110 hydrochloric acid in an ice bath for 5 minutes. Add 1 ml. of 4% sodium nitrite solution, mix, and keep in ice bath for at least 5 minutes before use.

To a purified sample in ice containing up to 0.025 mg. of histamine per ml., add 1 ml. of coupling buffer with continuous shaking or swirling. Let stand for 10 minutes in ice. Add 0.5 ml. of cold diazonium reagent and mix. After 20 minutes, add 5 ml. of methyl isobutyl ketone and shake vigorously 25 times. Remove from the ice and let stand at room temperature for 10 minutes to separate. Transfer 4 ml. of the clear upper layer into 5 ml. of barbital buffer. Shake vigorously 25 times. Add 1 ml. of 95% ethanol and shake vigorously 25 times. Let stand until all cloudiness disappears. If cloudiness is not visible, check by reading the solution at 5-minute intervals in the cuvet. Read at 490 $m\mu$ against methyl isobutyl ketone.

By p-sulfonic acid phenyldiazonium chloride. To prepare *p*-sulfonic acid phenyldiazonium chloride, dissolve 0.9 gram of sulfanilic acid monohydrate in 9 ml. of concentrated hydrochloric acid and dilute to 100 ml. To 2 ml. of this solution in ice, add 6 ml. of 6.2% sodium nitrite solution and dilute to 50 ml. with water. Keep in ice for 15 minutes before use. The reagent is stable for 12 hours.

To 5 ml. of 1.2% sodium carbonate solution in diethylenetriamine-pentaacetic acid, add 2 ml. of histamine solution containing $5 \times 10^{-6} M$

to 2×10^{-4} M of histamine. Mix and add 2 ml. of *p*-sulfonic acid phenyl-diazonium chloride solution. Mix quickly and read every 15 seconds at 500 $m\mu$ until a maximum absorbance is observed. This is approximately at the end of 2 minutes.

By 2,4-dinitrofluorobenzene. To prepare the carbonate buffer at pH 10, dissolve 21 grams of anhydrous sodium carbonate and 8.4 grams of sodium bicarbonate in a liter of water. To the sample solution, add 0.25 ml. of the buffer, and 0.02 ml. of 2% 2,4-dinitrofluorobenzene solution in absolute ethanol. Mix, and heat at 60° for 30 minutes. Add 0.4 ml. of methyl *n*-hexyl ketone. Mix vigorously, and centrifuge. Add a 0.2-0.35 ml. portion of the extract to 0.05 ml. of 1:5 hydrochloric acid. Mix for 10 seconds or long enough to produce the maximum fading. Centrifuge for 5 minutes. Remove the ketone layer and read the acid layer at 360 $m\mu$.

TRYPTAMINE, INDOLE-3-ETHYLAMINE, 3-(2-AMINOETHYL)-INDOLE

The condensation product of tryptamine with formaldehyde is oxidized to a highly fluorescent product, norharman.¹⁹⁶ Hydrogen peroxide is the oxidizing agent. Indolethylamine derivatives, such as indoleacetic acid, do not interfere. Tryptophan and 5-hydroxytryptamine do not interfere in concentrations less than 500 times that of tryptamine. The fluorescence from the reagents corrected by a blank is equivalent to 0.2-0.3 microgram of tryptamine.

The fluorescence of tryptamine may also be read in alkaline solution after extraction from the sample with benzene.¹⁹⁷ Since the tryptamine present in the final extract represents about 40% of that in the initial aqueous solution, standards of known amounts of tryptamine must be carried through the entire procedure. Beer's law is followed for 0.0001-0.008 mg. tryptamine.

Procedure—Urine. Adjust the pH of a 15-ml. sample to above 11 by the dropwise addition of 40% sodium hydroxide solution. Shake with 30 ml. of benzene for 20 minutes, and centrifuge. If an emulsion is present at the interface, break with a stirring rod and recentrifuge. Wash the

¹⁹⁶ Sidney M. Hess and Sidney Udenfriend, *J. Pharmacol. Exptl. Therap.* **127**, 175-7 (1959).

¹⁹⁷ Albert Sjoerdsma, John A. Oates, Perola Zaltzman and Sidney Udenfriend, *J. Pharmacol. Exptl. Therapy* **126**, 217-222 (1959); L. G. Allgen, K. E. Funke, and B. Nauckhoff, *Scand. J. Clin. Lab. Invest.* **13**, 390-5 (1961).

benzene layer with two 5-ml. portions of 0.4% sodium hydroxide solution to remove interferences, and centrifuge. Shake a 25-ml. aliquot of the benzene layer with 0.7 ml. of 1:110 hydrochloric acid for 15 minutes to return the tryptamine to the aqueous phase. Centrifuge, aspirate the benzene layer, and discard. Add a 0.5-ml. aliquot of the acid layer to 1 ml. of 0.13 *M* borate buffer at pH 10, and read tryptamine, using 285 $m\mu$ for activation and 360 $m\mu$ for fluorescence.

Tissue and urine. The sample should contain 0.0001-0.005 mg. of tryptamine per 3 ml. As a tissue sample, homogenize 1 gram with 2 ml. of 1:110 hydrochloric acid. Take a 3-ml. urine sample. Add 0.3 ml. of 40% sodium hydroxide solution to bring the pH to 11. Shake for 15 minutes with 20 ml. of benzene. Centrifuge, and shake the benzene with 5 ml. of 0.4% sodium hydroxide solution for 5 minutes. Centrifuge, and shake 15 ml. of the benzene layer with 4 ml. of 1:350 sulfuric acid for 5 minutes.

Centrifuge, and transfer 3 ml. of the acid layer to 0.1 ml. of 18% formaldehyde solution. Cover and heat for 20 minutes at 100°. Add 0.1 ml. of 5% hydrogen peroxide solution and heat at 100° for 20 minutes. Cool to room temperature and measure the fluorescence at 440 $m\mu$ with activation at 365 $m\mu$.

SEROTONIN, 5-HYDROXYTRYPTAMINE, 3-(2-AMINOETHYL)-5-INDOLOL

After extraction of 5-hydroxytryptamine into *n*-butanol from salt-saturated solutions, it may be read in the ultraviolet, by fluorescence, or by reaction with 1-nitroso-2-naphthol.¹⁹⁸⁻²⁰⁴ The absorption spectrum of 5-hydroxytryptamine in acid shows a maximum at 275 $m\mu$ and a smaller peak at 295 $m\mu$. Beer's law is followed up to 0.4 micromole per ml. at 275 $m\mu$. In the colorimetric method, with 1-nitro-2-naphthol, Beer's law is followed up to 0.8 micromole at 540 $m\mu$. The colorimetric method by 1-nitroso-2-naphthol has approximately the same sensitivity as the ultra-

¹⁹⁸ Sidney Udenfriend, Herbert Weissbach and Carroll T. Clark, *J. Biol. Chem.* **215**, 337-44 (1955).

¹⁹⁹ Donald F. Bogdanski, Alfred Pletscher, Bernard B. Brodie and Sidney Udenfriend, *J. Pharmacol. Exptl. Therap.* **117**, 82-8 (1956).

²⁰⁰ Michael Cais, *Med. Exptl.* **5**, 73-6 (1961).

²⁰¹ P. F. Crosti and P. E. Lucchelli, *Atti. Accad. Med. Lombarda* **16**, 104-8 (1961).

²⁰² Z. Orvids, E. Baumanis and J. Eiduss, *Lolrijas P S R Zinatnu Akad. Vestis* **9**, 79-83 (1962).

²⁰³ N. Crawford and B. T. Rudd, *Clin. Chim. Acta* **7**, 114-21 (1962).

²⁰⁴ W. B. Quay, *Anal. Biochem.* **5**, 51-59 (1963).

violet absorption method. The choice of procedure depends upon the concentration of ultraviolet-absorbing material present. In the absence of nitric acid, tyrosine will not react with 1-nitroso-2-naphthol. Tryptophan, tryptamine, 7-hydroxytryptophan, and 7-hydroxytryptamine do not form a color with the reagent, while *p*-hydroxyacetanilide does.

The concentration of 5-hydroxytryptamine in human blood is approximately 0.1-0.3 microgram per ml. To determine the amine in such small quantities, a fluorometric procedure is necessary. The apparatus consists of a xenon arc as a continuous light source and two quartz monochromators at right angles, one to select the activation energy and one to select the emitted fluorescence. An ultraviolet-sensitive photo-multiplier scanning device coupled to an oscillograph or automatic recorder produces records for analysis.²⁰⁵ Activation of 5-hydroxytryptamine occurs at 295 m μ . The maximum of the fluorescence spectrum occurs at 330 m μ or 540 m μ . Serotonin in deproteinized extracts of blood or tissues is separated on Amberlite CG-50 type 2 at pH 5 and eluted with 1:1 hydrochloric for fluorometric activation at 300 m μ and reading at 550 m μ .²⁰⁶

High recoveries of serotonin are obtained by extraction with *n*-butanol of an alkaline solution saturated with sodium chloride. From 40-80% of *N*-acetylserotonin, 5-methoxytryptamine, 5-hydroxytryptophan and bufotenine are also extracted. *N*-Acetylserotonin can be removed with diethyl ether before extraction. 5-Methoxytryptamine, bufotenine, and most of the 5-hydroxytryptophan can be removed by an additional wash with ether after an increase in pH. The following do not interfere: tryptophan, tryptamine, 5-methylindole, 3-methylindole, indole-3-acetic acid, 5-methyltryptophan, 4-hydroxy-*N*-dimethyltryptamine, *o*-phosphoryl-4-hydroxy-*N*-dimethyltryptamine, 4-hydroxytryptamine, 6-hydroxytryptamine, 4-hydroxytryptophan, and 6-methoxyindole.

When serotonin is acetylated, it turns a greenish blue color when treated with xanthidrol followed by hydrochloric acid. After extraction with acetic acid and methanol, the color is read at 640 m μ .²⁰⁷ Beer's law is followed for up to 0.1 mg. of serotonin-creatinine sulfate. Recoveries of added serotonin are only 80-83%, indicating losses during washing and elution from the carbon.

²⁰⁵ R. L. Bowman, P. A. Caulfield, S. Udenfriend, *Science* **122**, 32-3 (1955); P. I. Crosti and P. E. Lucchelli, *Atti. Accad. Med. Lombarda* **16**, 104-8 (1961); *J. Clin. Path.* **15**, 191-3 (1962).

²⁰⁶ V. E. Davis, J. A. Huff, H. Brown, and C. P. Alfrey, *Clin. Chim. Acta* **9**, 419-26 (1964); V. E. Davis, J. A. Huff, and H. Brown, *ibid.* **427-33**.

²⁰⁷ Klaus Gaedtke and Kurt Schreier, *Clin. Chem. Acta* **1**, 475-7 (1956).

Procedure—*By fluorescence. Blood.*²⁰⁸ *Histamine absent.* Hemolyze 1 ml. of blood with 6 ml. of water. Add 1 ml. of 6.3% sodium chloride solution. To deproteinize, add 1 ml. of 10% zinc sulfate solution followed by 0.5 ml. of 4% sodium hydroxide solution. After thorough shaking, centrifuge at 2500 rpm. for 20 minutes. Mix 1 ml. with 0.3 ml. of concentrated hydrochloric acid and read the fluorescence at 550 m μ by activating at 295 m μ .

Tissue. Homogenize with at least two volumes of 1:100 hydrochloric acid. Dilute an aliquot equivalent to no more than 2 mg. of tissue to 8 ml. with 0.9% sodium chloride solution. Proceed as for blood, from "To deproteinize . . ."

Histamine present. The foregoing procedures for blood and tissue report all hydroxyindoles. Therefore, if histamine is present, transfer another 5 ml. of the centrifugate. Prepare a salt mixture by drying trisodium phosphate at 100° for 48 hours, then at 300° for 2-3 hours. Finely powder and mix with anhydrous sodium sulfate in the ratio of 1:6.25.²⁰⁹

Add 1.8 gram of the salt mixture to the 5 ml. of clear solution. This brings the pH to about 12.5. Add 5 ml. of butanol and shake thoroughly for 5 minutes. Centrifuge at 2500 rpm for 15 minutes.

Prepare cotton acid succinate as follows: Dissolve 5 grams of anhydrous sodium acetate and 40 grams of succinic anhydride in 300 ml. of glacial acetic acid. Immerse 10 grams of cotton in this at 100° for 48 hours, with provision for exclusion of moisture. Filter the partially esterified cotton and wash successively with water, 1:10 hydrochloric acid, water, and 95% ethanol. Dry in a vacuum oven. From this, prepare a chromatographic column.

Pass the butanol layer through the column. Histamine is removed but serotonin goes through. Wash the column, after the butanol has gone through, with 3 ml. of 95% ethanol and 3 ml. of water. Discard these washings. Elute the histamine with 1 ml. of 1:100 hydrochloric acid followed by 0.5 ml. of water.

To the butanol, add 10 ml. of heptane and 1 ml. of 1:100 hydrochloric acid. Shake for 5 minutes and centrifuge to separate. Add 1 ml. of the acid layer to 0.3 ml. of concentrated hydrochloric acid and read serotonin fluorescently as previously described.

²⁰⁸ Herbert Weissbach, T. Philip Waalkes and Sidney Udenfriend, *J. Biol. Chem.* **230**, 865-71 (1958).

²⁰⁹ Floyd C. McIntire, L. W. Roth and J. L. Shaw, *J. Biol. Chem.* **170**, 537-44 (1947).

Platelets. Separate the platelets from the plasma by centrifuging for 20 minutes at under 4° . Resuspend in 1 ml. of water and add 0.2 ml. of 10% solution of zinc sulfate heptahydrate. Shake, and add 0.1 ml. of 4% sodium hydroxide solution. Shake for 1 minute, then centrifuge for 15 minutes. Mix 0.5 ml. of the clear layer with 0.15 ml. of concentrated hydrochloric acid. Activate at $295\text{ m}\mu$ and read the fluorescence at $550\text{ m}\mu$.

Tissue. The preparation of the sample appears under histamine, on page 147. To a 2-ml. aliquot, add 0.8 ml. of concentrated hydrochloric acid. Excite with a wave length of $300\text{ m}\mu$ to read the fluorescence at $550\text{ m}\mu$. The yellow fluorescence of serotonin is destroyed in 4-6 hours in 1:3 hydrochloric acid to give a blank correction.²¹⁰

In the presence of other 5-hydroxy and 5-methoxy indoles. To prepare the buffer for pH 10, dissolve 59.6 grams of boric acid and 41.7 grams of sodium hydroxide in 2 liters of water. Mix 0.1 ml. of 3% ascorbic acid solution in 1% ethylenediaminetetraacetic acid, 0.5 ml. of sample in 1:110 hydrochloric acid, 3 ml. of diethyl ether, and 300 mg. of sodium chloride. In another centrifuge tube, combine 8 ml. of heptane and 0.5 ml. of 1:110 hydrochloric acid containing 0.5% of ascorbic acid. Shake the first tube for 1 minute, centrifuge for 1 minute, and discard the ether phase containing *N*-acetylserotonin. Add another 3 ml. of ether and repeat the shaking, centrifuge, and discard the ether. Add 3 ml. of ether and 0.5 ml. of pH 10 buffer, which has been saturated with sodium chloride and diethyl ether. Shake for 1 minute, centrifuge for 1 minute, and discard the ether. Add 3 ml. of ether and repeat the shaking and centrifuging, and discard the ether. Immediately add 3 ml. of *n*-butanol, shake for 1 minute, centrifuge for 1 minute, and transfer the butanol phase containing serotonin to the second centrifuge tube containing the heptane. Shake for 2 minutes and centrifuge. Measure the volume of the aqueous phase. To a 0.25 ml. portion, add 0.075 ml. of concentrated hydrochloric acid and read the fluorescence at $540\text{-}550\text{ m}\mu$ with activation at $295\text{ m}\mu$.

By 2,4-dinitrofluorobenzene. Histamine. For histamine, mix the acid eluate provided for serotonin with 0.04 ml. of 0.002% solution of sodium diethyldithiocarbamate. Add 0.6 ml. of buffer for pH 10 containing 2.1% of sodium carbonate and 0.84% of sodium bicarbonate. Add 0.1 ml. of 2% 2,4-dinitrofluorobenzene in absolute ethanol. Heat at 60° for 30 minutes and cool. Add 1 ml. of 2-octanone and shake for 1 minute. Centrifuge.

²¹⁰ D. J. Boullin, *Life Sci.* **1**, 541-9 (1962).

remove 0.9 ml. of the ketone layer, and add 0.3 ml. of 1:5 hydrochloric acid. Mix by swirling and centrifuge. Read the acid layer at 360 $m\mu$.

Tissues. Purify *n*-butanol and heptane by shaking each with an equal volume of 0.4% sodium hydroxide solution and then with an equal volume of 1:110 hydrochloric acid. Shake twice with water. To prepare the borate buffer, dissolve 94.2 grams of boric acid in 3 liters of water. Add 165 ml. of 40% sodium hydroxide solution. Saturate the buffer with *n*-butanol and sodium chloride by adding the chemicals in excess and shaking. Remove excess *n*-butanol by aspiration and allow the excess salt to settle. The pH should be approximately 10.

Mix 1 ml. of borate buffer at pH 10, 2 grams of sodium chloride, and 15 ml. of *n*-butanol. Add 3 ml. of tissue extract, which has been adjusted to pH 10. Shake for 5 minutes and centrifuge to remove 5-hydroxytryptophan and tryptophan. Remove the aqueous phase by aspiration, add 15 ml. of borate buffer, and shake for 5 minutes. Remove the aqueous phase and add 15 ml. of buffer to the remaining phase. Shake for 5 minutes and centrifuge. Add a 10-ml. portion of the butanol layer to 20 ml. of purified heptane and 3 ml. of 0.5 *M* formate buffer at pH 4 to re-extract the 5-hydroxytryptamine into the aqueous phase. If to be developed in the visible range, substitute 1:600 hydrochloric acid for the buffer. Shake and centrifuge. Remove the supernatant solvent layer and develop the aqueous layer with 1-nitroso-2-naphthol, as below. Read in the ultraviolet at 275 $m\mu$ or read the fluorescence in the ultraviolet.

To prepare the nitrous acid reagent, add 0.2 ml. of 2.5% sodium nitrite solution to 5 ml. of 1:16 sulfuric acid. To 2 ml. of the acid extract containing 0.05-0.8 micromole of 5-hydroxytryptamine, add 1 ml. of 0.1% 1-nitroso-2-naphthol solution in 95% ethanol and 1 ml. of nitrous acid reagent. Stopper, shake, and heat at 55° for 5 minutes. Add 10 ml. of ethylene dichloride and shake to extract unchanged nitroso-2-naphthol reagent. Centrifuge at a low speed and read the supernatant aqueous layer at 540 $m\mu$.

*Separation of serotonin and 5-hydroxytryptophan.*²¹¹ The sample as in 1:100 hydrochloric acid. Pass a 2-ml. sample through a 2-cm. \times 2-mm. layer of 200-400 mesh Dowex 50W-X4 resin in sodium form. Wash the resin with 1 ml. of water. Elute the 5-hydroxytryptophan with 2 ml. of 0.02 *M* phosphate buffer for pH 9.2, collecting the eluate in 0.2 ml. of 2 *M* acetate buffer for pH 5. Read fluorescently at 296 to 342 $m\mu$.

²¹¹ R. G. Wiegand and L. Scherfling, *J. Neurochem.* **9**, 113-14 (1962).

Elute the 5-hydroxytryptamine with 2 ml. of 0.04% sodium hydroxide solution, collecting the eluate in 0.2 ml. of 1:50 hydrochloric acid. Read fluorescently at 300-342 $m\mu$.

By xanthydrol. Urine. Adjust a 100-ml. sample to pH 4 with acetic acid and pass through a column containing 20 grams of activated carbon. The particle size of the carbon is chosen so that the sample will pass through in 3 hours. Wash the column with 100 ml. of water and 200 ml. of a 4:1 mixture of ethanol and acetic acid to remove indoles and phenols. Dry the carbon in vacuum and acetylate the mixture by boiling for 4 hours with 30 ml. of acetic anhydride. Add 30 ml. of 95% ethanol and let stand for 10 minutes. Wash the carbon on a filter 5 times with 40-ml. portions of ethyl acetate, and evaporate the combined ethyl acetate extracts to near dryness *in vacuo* at 50°. Add 1.5 ml. of ethyl acetate and centrifuge the mixture. Dilute the supernatant brown liquid to a measured volume and take an aliquot for paper chromatography on Whatman No. 1 filter paper. As a developing solvent, use a 4:1:1 mixture of butanol-acetic acid-water. Dry the paper in air and spray with a 1:1 mixture of 10% xanthydrol solution in methanol and acetic acid. Dry, and bring the paper into contact with hydrochloric acid gas, which colors the spots of acetylated serotonin a greenish blue. Cut out the colored spots, extract with a 2:1 mixture of acetic acid and methanol, and read at 640 $m\mu$.

N-ACETYLSEROTONIN

N-Acetylserotonin is extracted into diethyl ether, and its fluorescence is read in a procedure similar to that used for serotonin.²¹² The melatonin, which is also extracted, is removed by cymene. 5-Hydroxy-indole-3-acetic acid and bufotenine are excluded by use of a double extraction with ether. At a higher pH, 5-hydroxyindole-3-acetic acid is excluded, and at an acid pH, bufotenine is excluded.

Procedure—Follow the procedure for serotonin in the presence of other 5-hydroxy and 5-methoxy indoles, using *p*-cymene as the solvent in place of ether. Start at the beginning and continue through "In another centrifuge tube combine 8 ml. of heptane and 0.5 ml. of 1:110 hydrochloric acid containing 0.5% of ascorbic acid." Shake the tube containing the sample for 1 minute, centrifuge for 1 minute, and discard the cymene to remove melatonin. Add another 3 ml. of cymene and repeat the shaking

²¹² W. B. Quay, *Anal. Biochem.* **5**, 51-59 (1963).

and centrifuging. Discard the cymene. Add 3 ml. of diethyl ether, shake 1 minute, and centrifuge to extract *N*-acetylserotonin. Transfer the ether phase to the tube containing heptane. Shake 2 minutes and centrifuge. Transfer the aqueous phase to a centrifuge tube containing 300 mg. of sodium chloride and 3 ml. of diethyl ether. Add 0.25 ml. of pH 10 buffer saturated with sodium chloride and diethyl ether. Immediately shake for 1 minute, centrifuge for 1 minute, and transfer the ether phase to a centrifuge tube containing 8 ml. of heptane and 0.5 ml. of 1:110 hydrochloric acid containing 0.5% of ascorbic acid.

Shake for 2 minutes and centrifuge. Measure the volume of the aqueous phase and read the fluorescence at 540-550 $m\mu$ with activation at 295 $m\mu$.

MELATONIN, *N*-ACETYL-5-METHOXYTRYPTAMINE

Selective extraction of melatonin occurs with *p*-cymene from an alkaline solution. The fluorescence is read at 540-550 $m\mu$.²¹³

Procedure—Follow the procedure under serotonin (page 157), starting at the beginning and ending with "In another centrifuge tube, combine 8 ml. of heptane and 0.5 ml. of 1:110 hydrochloric acid containing 0.5% of ascorbic acid," replacing the diethyl ether with *p*-cymene. To the tube containing the sample, add 0.25 ml. of pH 10 buffer saturated with sodium chloride and *p*-cymene. Immediately shake for 1 minute, centrifuge for 1 minute, and transfer the cymene phase to the tube containing heptane. Shake for 2 minutes and centrifuge. Measure the volume of the aqueous phase and read the fluorescence at 540-550 $m\mu$ with activation at 295 $m\mu$.

3-METHOXYTYRAMINE

3-Methoxytyramine on oxidation with iodine and irradiation gives a fluorescent reaction. It is separated from adrenaline, noradrenaline, and dopamine by chromatography.²¹⁴

Procedure—Set up a 120 × 3-mm. chromatographic column of Dowex 50W-X8. Wash it with 40 ml. of 1:5 hydrochloric acid and then with water until the washings are neutral. Treat the column with 30 ml. of 0.1 *M* phosphate buffer for pH 6.5.

²¹³ *ibid.*

²¹⁴ A. Carlsson and B. Waldeck, *Scand. J. Clin. Lab. Invest.* **16**, 133-S (1964).

To 5-20 ml. of perchloric acid extract of tissue, add 0.1 gram of ethylenediamine tetraacetate. Adjust the pH to 7 with 35% potassium carbonate solution. Refrigerate and centrifuge. Transfer the clear upper layer to the column. Wash the precipitate with a few ml. of ice water and add this to the column. Elute the column with 1:10 hydrochloric acid. Adrenaline and noradrenaline come through in the first 12 ml. The next 24 ml. contain dopamine. Now elute the 3-methoxytyramine with 15 ml. of 1:5 hydrochloric acid.

As a buffer for pH 6.5, mix 12 ml. of 19.2% citric acid solution with 12 ml. of 14.2% solution of anhydrous disodium phosphate. Adjust the eluate to pH 6.5 with 35% potassium carbonate solution. Take an aliquot equivalent to 0.1-1 microgram of test substance. Add 0.5 ml. of the buffer and dilute with water to 3.7 ml. Add 0.1 ml. of 0.25% iodine solution. After 7 minutes, add 0.5 ml. of alkaline sodium sulfite solution. To prepare this, dissolve 5.04 grams of sodium sulfite heptahydrate in 10 ml. of water and dilute to 100 ml. with 20% sodium hydroxide solution. After another 5 minutes, add 0.7 ml. of 1:1 hydrochloric acid. Irradiate with a mercury-vapor lamp around 254 m μ for 15 minutes. Activate the fluorescence at 335 m μ and read at 385 m μ . As a reagent blank, develop 2 ml. of 11.2% potassium chloride solution, and as a tissue blank omit the addition of iodine solution.

GLUTETHIMIDE, 2-ETHYL-2-PHENYLGLUTARIMIDE

Glutethimide, a nonbarbiturate sedative, may be determined after chloroform extraction by the characteristic ultraviolet spectrum and by the rate of hydrolysis in alcoholic potassium hydroxide.²¹⁵ This method is specific, rapid, and detects less than 0.002 mg. per ml. with an error of approximately 5%.

Glutethimide cannot be read in the ultraviolet in dilute alkaline solution, since there is rapid hydrolysis with opening of the piperidine ring. Reading is possible in alcoholic potassium hydroxide with a maximum at 233 to 235 m μ and a minimum at 223-225 m μ . Beer's law is followed up to 0.025 mg. per ml. 2-Phenylglutarimide and 3-methyl-3-phenylglutarimide do not interfere.

A colorimetric method based on the formation of a hydroxamic acid derivative with alkaline hydroxyl amine estimates glutethimide in the range of 0.5-7 micromoles per 5 ml. and its metabolite, α -phenylglutari-

²¹⁵ Leo R. Goldbaum, Melvin A. Williams and Theodore Koppányi, *Anal. Chem.* **30**, 81-4 (1960).

imide, in the same concentration range.²¹⁶ The maximum color develops in 30 minutes and remains stable for over 2 hours. Decomposition occurs over 60°. Ferric chloride is added to influence the stability and intensity of the color.

In the presence of fatty materials, such as in vomitus samples, a turbidity forms with glutethimide in ethanolic potassium hydroxide.²¹⁷ Fatty materials containing glutethimide may be determined with hydroxylamine hydrochloride by extraction of the purple color formed into isobutanol. The amount of ferric chloride is decreased to give less color to the isobutanol. Barbiturates and brominated ureides do not interfere. Glutethimide reacts in concentrated sulfuric acid containing formaldehyde to give a fluorescent compound.²¹⁸

Sample—*Blood, plasma or urine.* Extract a 1-5 ml. sample containing up to 0.025 mg. of glutethimide with 25 ml. of chloroform. Shake vigorously for 1 minute and allow the layers to separate. Aspirate off the sample and discard. Run the chloroform through fast filter paper and wash twice by shaking with 5-ml. portions of 2% sodium hydroxide solution to remove acidic substances extracted by the chloroform. Aspirate each wash. Shake the chloroform extract with 5 ml. of 1:24 hydrochloric acid to remove basic substances extracted. Aspirate off the wash. Filter the chloroform and evaporate a 20-ml. aliquot at 100° to approximately 2-3 ml. Air dry and develop in alcoholic potassium hydroxide.

*Serum.*²¹⁹ Add 1 ml. of water and 21 ml. of ethyl acetate to 1 ml. of sample. Shake for 5 minutes and centrifuge for 1 minute. Discard the aqueous layer and filter the ethyl acetate layer through paper into a centrifuge tube. Wash this successively with 5 ml. of 0.8% sodium hydroxide solution and with 5 ml. of 1:22 hydrochloric acid. Dry with 0.5 gram of anhydrous sodium sulfate.

Mix 14 ml. of the dried ethyl acetate solution with 25 mg. of activated carbon and boil gently. Filter and evaporate the filtrate to 3 ml. Filter, wash the beaker and filter with ethyl acetate, and evaporate the filtrate

²¹⁶ Herbert Sheppard, Barbara S. D'Asaro and Albert J. Plummer, *J. Am. Pharm. Assoc.* **45**, 681-4 (1956).

²¹⁷ S. E. Phang, M. C. Dutt and Thug Soon Tee, *J. Pharm. and Pharmacol.* **13**, 319-20 (1961).

²¹⁸ R. P. Haycock, P. B. Sheth and W. J. Mader, *J. Am. Pharm. Assoc., Sci. Ed.* **49**, 673-7 (1960).

²¹⁹ T. Vos, *Pharm. Weekbl.* **97**, 517-24 (1962).

to dryness at 50°. Develop with alcoholic potassium hydroxide after the odor of ethyl acetate has disappeared.

Urine. Free glutethimide. Shake a 25-ml. sample with 25 ml. of benzene. Remove the benzene and re-extract the urine with 15 ml. of benzene. This aqueous phase is the sample for conjugated glutethimide below.

Combine the benzene extracts and concentrate them to approximately 5 ml. *in vacuo* with the aid of some heat. Dry the residual extract with anhydrous sodium sulfate.

To prepare the column, place 1 gram of aluminum oxide, previously dried *in vacuo*, in a 25-ml. buret filled to the top with dried benzene. Fit with a teflon stopcock and a glass wool plug above the stopcock. Allow the aluminum oxide to settle and place a glass wool plug at the top. Wash the column with the benzene contained in the buret. Add the sample and wash the column with an additional 25 ml. of benzene. Discard the benzene eluates. Add 25 ml. of a 9:1 chloroform-methanol mixture, which has been previously dried with sodium sulfate. Collect 25 ml. of eluate. Evaporate the chloroform-methanol eluate to dryness. Develop with hydroxylamine hydrochloride.

Conjugated glutethimide or α -phenyl glutarimide. Reflux the aqueous phase remaining after the above benzene extraction with 3.5 ml. of concentrated hydrochloric acid for 6 hours to completely hydrolyze the conjugated glutethimide. Cool, and follow the preparation technic for free glutethimide, starting with the extraction with 25 ml. of benzene. Use approximately 5 grams of aluminum oxide. All glutethimide should be in the first 25 ml. of chloroform-methanol.

Whole blood. Extract the sample with ethyl acetate. Evaporate the ethyl acetate *in vacuo* and take up the residue in benzene. Follow the procedure for urine, starting at "To prepare the column . . ."

Vomitus and biological samples containing large quantities of fatty material. Extract the sample with chloroform. Filter and evaporate the solvent to 2-3 ml. with heat and to dryness at room temperature. Dissolve the residue in methanol, so that 1 ml. contains up to 1 mg. of glutethimide. Develop with hydroxylamine hydrochloride and isobutanol.

Procedure—*By alcoholic potassium hydroxide.* To the dry residue of a sample containing up to 0.025 mg. of glutethimide, add 3.2 ml. of ab-

solute ethanol. Mix well and transfer 2.4 ml. to a 1-cm. cell. Add 0.8 ml. of 1.12% potassium hydroxide solution. Mix, and read against a reagent blank at 1, 2, 3, 4, and 5 minutes. Add 0.2 ml. of 8.56% ammonium chloride solution. This adjusts the pH to 9.7. Read this and subtract from the previous values. Extrapolate the readings at 1-5 minutes to zero time.

By hydroxylamine hydrochloride. Dissolve the dry residue from a sample containing 0.5-7 micromoles of glutethimide per 5 ml. in 1 ml. of absolute methanol. Add 1 ml. of 1.4% hydroxylamine hydrochloride solution and 1 ml. of 14% sodium hydroxide solution. Mix, and let stand at room temperature for 30 minutes. Add 1 ml. of 1:2 hydrochloric acid and mix. Add 1 ml. of 6% ferric chloride solution in 1:110 hydrochloric acid. Mix, and read within 5 minutes at 540 m μ .

By hydroxylamine hydrochloride and isobutanol. To 1 ml. of methanol solution containing 0.25-1 mg. of glutethimide, add 1 ml. of 1.4% hydroxylamine hydrochloride solution and 1 ml. of 14% sodium hydroxide solution. After 30 minutes, add 1.5 ml. of 1:2 hydrochloric acid, 5 ml. of isobutanol, and 0.5 ml. of 6% ferric chloride solution in 1:110 hydrochloric acid. Shake vigorously for 30 seconds and let the layers separate. Filter the isobutanol layer and read immediately at 510 m μ against a reagent blank.

D-PANTHENOL, 2,4-DIHYDROXY-N-(3-HYDROXYPROPYL)3-3-DIMETHYLBUTYRAMIDE

D-Panthenol in complex multivitamin preparations is developed by 1,2-naphthoquinone-4-sulfonate²²⁰ (cf. Vol. IV, p. 51). The solution is saturated with ammonium sulfate and the panthenol extracted into benzyl alcohol, leaving most interferences in the aqueous phase. The D-panthenol is then forced into water by addition of toluene which reduces the solubility of the D-panthenol in benzyl alcohol.

Panthenol and pantothenates are hydrolyzed to β -alanol or β -alanine. The amino nitrogen group is then determined by 1,2-naphthoquinone-4-sulfonate or by ninhydrin²²¹ (cf. Vol. IV, p. 51). As little as 2.5 mg. of panthenol or pantothenates can be measured in multivitamin prepara-

²²⁰ Thavil Panalaks and J. A. Campbell, *Anal. Chem.* **33**, 1038-40 (1961).

²²¹ Morton Schmall and Ernest G. Wollish, *ibid.* **29**, 1509-13 (1957).

tions by the naphthoquinone method. The ninhydrin method measures as little as 1 mg. and is a more rapid and sensitive procedure.

Riboflavin is eliminated by passage through a double-bed column of Dowex 50-X4 and Florasil. Riboflavin 5'-phosphate or molybdates are removed with an anion exchange column consisting of Dowex 1-X4 (Cl-type) or Amberlite IRA-100 (OH or Cl type). A Dowex 50-X4 (H-type) column is used to eliminate other interferences, which include niacinamide, all amino acids, liver extracts, ferrous and ferric iron, copper, tin, zinc, manganese, and cobalt. Sugars, ascorbic acid, and the emulsifiers—Tweens and Spans—do not interfere. In samples where all these interferences appear, a triple-bed column consisting of a cation exchanger, Dowex 50(H), an anion exchange resin, and an adsorbent such as Florasil is employed.

Sample—*Pharmaceutical preparations and multivitamin preparations.* To prepare the resin, add a volume of 8% sodium hydroxide solution approximately twice that of the Dowex 50-X12, H-form, 50-100 mesh. Stir, and let stand for 30 minutes. Wash with water three times by decantation. Add a volume of 1:5 hydrochloric acid approximately equal to the volume of sodium hydroxide solution used. Stir, and let stand for 30 minutes. Wash with water by decantation until the washings are neutral to litmus. Store the resin under water. To prepare Amberlite IRA-400, OH-form, 20-50 mesh, follow the procedure for Dowex 50-X12, reversing the order of alkali and acid washing.

Place a glass wool plug at the bottom of a 10-mm \times 30-cm chromatographic tube and add Amberlite resin to a height of 7 cm. Cover with glass wool and add Dowex resin to a height of 7 cm. Shake 10 ml. of an aqueous extract of a sample containing 2-4 mg. of D-panthenol with 7 grams of ammonium sulfate for 5 minutes. Add 20 ml. of benzyl alcohol. Shake mechanically for 15 minutes. Pipet 10 ml. of the upper benzyl alcohol layer into 10 ml. of toluene and add 15 ml. of water. Shake for 15 minutes and centrifuge. Pipet 10 ml. of the lower aqueous layer containing panthenol into the chromatograph column, allowing the solution to pass through rapidly. Collect the effluent and wash the column with three 5-ml. portions of water or until the total volume of effluent and washings is 25 ml. Develop by the first method, using 1,2-naphthoquinone-4-sulfonate.

As an alternative technic for samples in which riboflavin is not present, use a 12-mm. \times 20-cm. chromatographic column. Place approximately 3 cm. of Dowex-X4 (H-type) 100 to 200 mesh resin between two small

pledgets of glass wool. For samples containing small amounts of riboflavin, place 3 cm. of 60 to 100 mesh Florasil below the Dowex 50, separated from it by a glass wool plug. If large quantities of riboflavin are present, increase the length of the Florasil section to 5 cm. In the presence of riboflavin 5-phosphate or molybdates, a 3-cm. section of Dowex 1-X4 (Cl-type) or Amberlite IRA-400 (OH- or Cl-type) should be inserted between two glass wool plugs between the Florasil and Dowex 50. Wash the entire column with 20 ml. of water. Dissolve the sample so that a 25-ml. aliquot contains 2.5-5 mg. of panthenol or panthenate. Add the sample to the column filled with water and adjust the flow to a rate of 0.5 ml. per minute. When the sample reaches the level of the top of the resin, elute rapidly with water so that the eluate is approximately 40-45 ml. Develop the eluate with 1,2-naphthoquinone-4-sulfonate, using the second technic under that heading.

Alternatively, prepare the chromatographic column as described above, ending with "Wash the entire column with 20 ml. of water . . ." Dilute a sample so that a 25 ml. aliquot contains 1-2 mg. of panthenol or panthenate. Add the sample to the column and adjust the flow rate to approximately 0.5 ml. per minute. When the sample reaches the top of the resin, elute rapidly by adding enough water to bring the volume of the eluate to 40 ml. Develop with ninhydrin.

Procedure—*By 1,2-naphthoquinone-4-sulfonate.* This is applicable to the purified extract for determination without hydrolysis. To prepare borate buffer, dissolve 75 grams of sodium hydroxide in 500-600 ml. of water and add 150 grams of sodium tetraboratedecahydrate. Dilute to 1 liter. Divide a 25-ml. effluent into two 12.5 ml. portions. To each, add 1 ml. of 1:5 hydrochloric acid and autoclave at 15 pounds for 30 minutes. Cool to room temperature.

To flask A, add 1 ml. of borate buffer and 1 ml. of a 0.5% solution of the sodium salt of 1,2-naphthoquinone-4-sulfonate. To flask B, add 1 ml. of borate buffer. Prepare a third flask C as a reagent blank by adding 12.5 ml. of water, 1 ml. of 1:5 hydrochloric acid, 1 ml. of borate buffer, and 1 ml. of naphthoquinone reagent. Heat all the flasks at 100° for 10 minutes. Cool to room temperature and add 0.5 ml. of 1.6% sodium thiosulfate solution to each. Let stand for 10-30 minutes and dilute each to 25 ml. with water. Read at 465 $m\mu$ against water. Correct the net absorbance for the sample and reagent blanks.

For this second technic, the sample should be free of riboflavin and

other interferences, and should contain 2.5-5 mg. of panthenol or panthenate in 40-45 ml. of water.

To prepare the formaldehyde reagent, combine 3 parts of 1:7 hydrochloric acid, 1 part of glacial acetic acid, and 1 part of 0.45% formaldehyde solution. Heat the sample with 3 ml. of 10% sodium hydroxide solution at 100° for 1 hour to ensure complete cleavage to β -alanol or β -alanine. Cool to room temperature and dilute to 50 ml. with water. To a 5-ml. aliquot, add 1 drop of phenolphthalein reagent and 0.6 ml. of 1:35 sulfuric acid. Titrate with 1:350 sulfuric acid to a colorless end point.

The addition of 1 ml. of 1% borax solution should turn the solution pink. If not, titrate another 5-ml. aliquot. Add 1 ml. of 0.5% 1,2-naphthoquinone-4-sulfonate solution and heat at 100° for 10 minutes. Cool to room temperature and add 1 ml. of formaldehyde reagent and 1 ml. of 2.5% sodium thiosulfate. After at least 10 minutes, dilute to 25 ml. with ethanol to clear any turbidity present. Read at 465 m μ against a reagent blank. If the solution is colored or turbid after hydrolysis, it may be centrifuged. To eliminate the interference of color, prepare a sample blank without the naphthoquinone reagent. Set the photometer at 0 absorbance with this blank. To a 5-ml. aliquot of the hydrolyzed sample, add all reagents except naphthoquinone. In another flask, combine all reagents. Subtract the sum of the absorbances of the last two solutions from the reading of the sample.

By ninhydrin. The sample should be free of riboflavin and other interferences and should contain 1-2 mg. panthenol or panthenate in 40 ml. of water. To prepare the phenol solution, dissolve 80 grams of phenol in 20 ml. of absolute ethanol with gentle heating. Shake with 1 gram of Dowex 50 (H-type) for 20 minutes to remove any traces of ammonia and decant. To prepare the cyanide-pyridine reagent, dilute 2 ml. of 0.065% freshly prepared potassium cyanide solution to 100 ml. with ammonia-free pyridine, which has been prepared by shaking 100 ml. of pyridine with 1 gram of Dowex 50 (H) for 20 minutes.

Heat the sample with 5 ml. of 1% sodium hydroxide solution at 100° for 1 hour. Cool to room temperature. Titrate with 1:35 sulfuric acid to the phenolphthalein colorless end point, adding 2 drops of acid in excess. Add 50 ml. of ethanol and dilute to 100 ml. with water. To a 2-ml. aliquot, add 1 ml. of phenol reagent and 1 ml. of pyridine-potassium cyanide reagent. Heat at 95-100° for 1 minute and add 0.2 ml. of 5% ninhydrin solution in absolute ethanol. Stopper, and heat at 95-100° for 5 minutes.

Cool to room temperature and dilute to 10 ml. with 60% ethanol. Prepare a reagent blank using 2 ml. of 60% ethanol in place of the sample. Read the sample at 570 $m\mu$ against the reagent blank. To compensate for color in the sample, follow the technic described under the second method for naphthoquinone, substituting ninhydrin for naphthoquinone.

PANTOTHENATES, SALTS OF 2,4-DIHYDROXY-N-(2-CARBOXYETHYL)-
3,3-DIMETHYL-BUTYRAMIDE

In both acid and alkaline medium, calcium pantothenate is cleaved to the hydroxy acid, α,γ -dihydroxy- β,β -dimethylbutyric acid. In the presence of sulfuric acid, the hydroxy acid reacts with 2,7-naphthenediol to yield a colored complex which is read at 465 $m\mu$.²²² Large amounts of thiamine, pyridoxine, choline, niacin, niacinamide, vitamins A and D, vitamin B₁₂, α -tocopherol, citric acid, tartaric acid, and β -alanine do not interfere. Riboflavin is removed by passage through Florasil. Treatment with calcium hydroxide and copper sulfate removes ascorbic acid, glucose, and lactose. Beer's law is followed up to 0.5 mg.

Sample—*Multivitamin preparations and animal feed supplements.* To prepare the Florasil, cover 100-200 grams of 60-100 mesh material with 2% acetic acid-20% pyridine mixture. Boil gently for several minutes with constant stirring and allow to settle. Decant the supernatant liquid and repeat the washing and decanting twice. Wash the Florasil with hot water to remove solvent, and dry in an oven at 100-120°. To a column 12 mm. \times 50 cm., constricted at the lower end, slowly add 4 grams of Florasil on top of a glass wool plug. Apply gentle suction and wash the column with 25 ml. of water.

To a sample containing 100 mg. of calcium pantothenate, add 100 ml. of water. Shake for at least 10 minutes. Centrifuge or filter. Add 25 ml. of the filtrate to the Florasil column as described. Collect the eluate and wash the column with a total of 25 ml. of water in small portions. To the combined eluate and washings, add 1 gram of anhydrous cupric sulfate. Stopper, and shake to dissolve. Add 3 grams of calcium hydroxide. Shake, and let stand for 30 minutes with occasional shaking. Filter through a sintered glass filter. Wash the flask and filter with 25 ml. of water. Develop the combined filtrate and washing with naphthalenediol.

²²² C. R. Szalkowski and J. H. Davidson, *Anal. Chem.* **25**, 1192-5 (1953).

Procedure—By 2,7-naphthalenediol. To prepare the reagent, dissolve 500 mg. of 2,7-naphthalenediol in 500 ml. of concentrated sulfuric acid and let stand 18-24 hours or until practically colorless. Store protected from light.

To a sample containing up to 25 mg. of calcium pantothenate, add 5 ml. of concentrated sulfuric acid. Reflux gently for 1 hour. Cool to room temperature and dilute to 100 ml. with water. Place a 1-ml. aliquot in a test tube and place in ice for 5 minutes. Add 10 ml. of 2,7-naphthalenediol reagent. Mix by careful shaking and heat at 100° for 30 minutes. Cool in ice for 5 minutes and add 15 ml. of 1:1 sulfuric acid. Mix thoroughly and after 30 minutes read at 465 $m\mu$ against a reagent blank.

By 1,2-naphthoquinone-4-sulfonate. See panthenol, page 166.

By ninhydrin. See panthenol, page 167.

HYDRAZINE AND METHYLHYDRAZINES

Hydrazine reduces phosphomolybdic acid to molybdenum blue, which is read at 420 $m\mu$ ²²³ (cf. Vol. IV, p. 54). Beer's law is followed up to 0.035 mg. of hydrazine, to 0.035 mg. of methylhydrazine, and to 0.055 mg. of *s*-dimethylhydrazine. By increasing the heating time in the method to 50 minutes, Beer's law is followed for *as*-dimethylhydrazine for concentrations up to 0.05 mg.

A turbidimetric micromethod for hydrazine is based on the reduction of selenium oxide in acidic solution and reading the resulting turbidity of the selenium.²²⁴ An indirect method for the analysis of hydrazine is based on its oxidation with an excess of potassium chromate and the subsequent determination of the excess by reaction with *o*-dianisidine in acid medium.²²⁵ Any substances that are oxidized by chromate interfere. This method is also applicable to hydroxylamine.

Hydrazine is also determinable by reaction with *p*-dimethylaminobenzaldehyde to form *p*-dimethylaminobenzaldazine.²²⁶ The product rearranges in the presence of a strong acid to form a *p*-quinoic chromo-

²²³ Leo Fensilver, Joseph A. Perregrino and Courtney J. Smith, Jr., *Am. Ind. Hyg. Assoc. J.* **20**, 26-31 (1959).

²²⁴ M. R. F. Ashworth, *Microchim. Acta* **5-10** (1961).

²²⁵ F. Buscarións, J. Artigas and C. Rodríguez-Roda, *Anal. Chim. Acta* **23**, 214-16 (1960).

²²⁶ Thomas Danbrauskas and Herbert H. Cornish, *Am. Ind. Hyg. Assoc. J.* **23**, No. 2, 151-6 (1962).

phore, sensitive to 0.00001 mg. per ml. of the final solution. Hydrazine is also reacted with 4-(dimethylcarbamoyl)benzaldehyde for reading at not less than 0.01 mg. per liter.²²⁷ Ammonium chloride does not interfere.

Trisodium pentacyanoaminoferoate reacts with 1,1-dimethylhydrazine to form a red complex for reading at 540 m μ .²²⁸ The pH should be 4-6, suitably adjusted by treatment with a citric acid-phosphate buffer for pH 5.4. The full color develops in one hour and is stable for 3 hours thereafter. The method will detect 1-60 gamma per ml. or 2.5-50 ppm. in air.

Hydrazine and the primary hydrazides are determined by their effect on ferric 2,2'-bipyridyl.²²⁹ Beer's law applies from 0.1-0.5 micromole. Hydrazine forms a fluorescent aldazine with 2-hydroxy-1-naphthaldehyde. The maximum fluorescence at 534 m μ is linear over a range of 0.02-0.8 microgram per ml.²³⁰ A general reaction of hydrazine, monoalkyl hydrazines, and both symmetrical and unsymmetrical dialkylhydrazines is to reduce 1,2-naphthoquinone-4-sulfonic acid to 1,2-dihydroxynaphthoquinone-4-sulfonic acid.²³¹ The latter is then activated at 340 m μ to give fluorescence at 470 m μ .

Procedure—*By reduction of phosphomolybdic acid.* As a reagent, dissolve 40 grams of sodium hydroxide in 400 ml. of water. Add 70 grams of molybdic acid and 10 grams of sodium tungstate. Boil for 30 minutes, cool, and dilute to 700 ml. Add 250 ml. of 85% phosphoric acid, dilute to 1 liter and mix.²³²

Add 2 ml. of phosphomolybdic acid reagent to a solution of hydrazine salts containing up to 0.035 mg. of hydrazine, up to 0.035 mg. of methylhydrazine, up to 0.055 mg. of *s*-dimethylhydrazine, and up to 0.05 mg. of *as*-dimethylhydrazine. Dilute to 8 ml. and heat 15 minutes at 98° for all salts except *as*-dimethylhydrazine, which requires a heating time of

²²⁷ M. T. Dmitriev, *Trudy Nauch-Issled. Inst. Gidrometeorol. Priborostr.* 1963 (11), 62-6.

²²⁸ Mildred K. Pinkerton, Jay M. Lauer, P. Diamond and Anton A. Tamas, U.S. Dept. Com., Office Tech. Services A.O. 273, 986, 10 pp. (1961).

²²⁹ F. B. Wheatley, M. L. Ashby and C. L. Mehlretter, *Microchem. J.* 7, 185-93 (1963).

²³⁰ Tsutomu Momose, Yo Ueda, Yoshiko Mukai and Kyoko Watanabe, *J. Pharm. Soc. Japan* 80, 225-8 (1960).

²³¹ M. Roth and J. Rieder, *Anal. Chim. Acta* 27, 20-26 (1962).

²³² C. C. Comstock, L. H. Lawson, E. A. Greene and F. W. Oberst, *Am. Med. Assoc. Arch. Ind. Hygiene and Occupational Medicine* 10, 478 (1954).

50 minutes. Cool rapidly to room temperature. Dilute to 10 ml. with water and read at 420 m μ .

Turbidimetrically by selenium oxide. Heat a solution containing 0.006-0.06% of hydrazine for 1-10 minutes with an equal volume of a solution containing 0.04% of selenium oxide and 0.4% of polyvinyl pyrrolidinone solution made to 60% with acetic acid. Read the turbidity of the reduced selenium at 520 m μ .

By potassium chromate. To prepare the reagent, dissolve 0.5 gram of *o*-dianisidine in 50 ml. of acetone and dilute to 100 ml. with water. To 2 ml. of 20% potassium chromate solution, add a 2-ml. sample containing up to 4 ppm. of hydrazine and 3 ml. of 1:6 sulfuric acid. After 5-10 minutes, add 0.5 ml. of *o*-dianisidine reagent and read at 470 m μ between 5 and 30 minutes after reagent addition.

By ferric bipyridyl. As a reagent, dissolve 1 gram of ferric chloride in 20 ml. of concentrated hydrochloric acid and dilute to 1 liter. Add 1 ml. to 10 ml. of sample solution containing about 0.3 micromole of hydrazine or the hydrazide. As a buffer, dissolve 8.3 grams of sodium acetate trihydrate in water, add 12 ml. of glacial acetic acid, and dilute to 100 ml. with water. Add 5 ml. of this buffer and mix. Add 2 ml. of 1% aqueous 2,2'-bipyridyl and mix. Heat to 85° to obtain the maximum color. This requires from 50 minutes for methylhydrazine to 120 minutes for hydrazine hydrate. Cool to 25°, dilute to 25 ml. and read at 510 m μ .

By 4-(dimethylcarbamoyl)benzaldehyde. Air. Draw the sample at 1 liter per minute through a 200-ml. column containing 50 ml. of water with silica gel suspended in it. After 10 minutes, filter and add 1 ml. of 1:2 hydrochloric acid. Add 1 ml. of 0.2% solution of the reagent and read at 450-470 m μ .

METHYLHYDRAZINE

Reaction of methylhydrazine with *p*-dimethylaminobenzaldehyde is similar to that of hydrazine with the same reagent²³³ (Vol. IV, p. 54). To eliminate errors from reagent impurity, it is necessary to run a standard methylhydrazine determination with each reagent solution. If the

²³³ H. Bert McKennis, Jr. and Allan S. Yard, *Anal. Chem.* **26**, 1960-3 (1954).

reagent is pure, the procedure is accurate to 1.5% in the region of 0.025-0.08 mg. of methylhydrazine sulfate.

Aromatic amines, urea, adrenalone, and semicarbazide interfere. Mono-, di-, and trimethylamines, di- and triethylamines, ethanolamine, glycocoll, choline, urethane, aminoguanidine, hydroxylamine, ammonium chloride, and ammonium nitrate do not interfere. Hydrazine must be absent. Beer's law is followed at 458 $m\mu$. Methylhydrazine may also be developed by reduction of phosphomolybdic acid as given under hydrazine.

Procedure—To prepare *p*-dimethylaminobenzaldehyde reagent, dissolve 0.2 gram in 5 ml. of 1:16 sulfuric acid.²³⁴ Dilute a sample containing 0.025-0.08 mg. of methylhydrazine sulfate to 5.5 ml. with water. Add 0.5 ml. of reagent and adjust the volume to 6 ml. Read after 15 minutes against a blank.

UNSYMMETRICAL DIMETHYLHYDRAZINE

A convenient method of determining unsymmetrical dimethylhydrazine in aqueous solution in the ppm. range is to oxidize to formaldehyde and develop with chromotropic acid.²³⁵ The reaction is quantitative and therefore permits the use of a formaldehyde curve for interpretation. There is no interference by 500 ppm. of chloride, 250 ppm. of sulfate, 0.05 ppm. of hexavalent chromium, 5 ppm. of fluoride or 1 ppm. of boron. Nitrate produces a yellow color which interferes with visual reading but not with spectrophotometric. This can be avoided by adding 2 drops of concentrated hydrochloric acid and 1 ml. of concentrated sulfuric acid per ml. of sample just prior to addition of the chromotropic acid. Up to 500 ppm. of ethanol or of hydrazine do not interfere. Monomethylhydrazine reacts much like the unsymmetrical dimethyl compound.

Procedure—*Aqueous solutions.* Heat 200 ml. of sample containing about 50 ppm. of unsymmetrical dimethylhydrazine at 90° for 20 hours under a reflux condenser. A lesser time will give low results. Rinse down the walls with the refluxed sample. To an appropriate portion of the sample, usually 0.25-1.5 ml., add 10-70 mg. of solid chromotropic acid. Cautiously add concentrated sulfuric acid to make a volume of 50 ml. Heat at 60-70° for 10 minutes and cool. Read at 580 $m\mu$. Interpret from

²³⁴ Paul R. Wood, *ibid.* **25**, 1879-83 (1953).

²³⁵ Norma V. Sutton, *Anal. Chem.* **36**, 2118-19 (1964).

a formaldehyde curve, and since the molecular weight of the dimethylhydrazine is exactly twice that of formaldehyde, multiply the result by two.

HEA, OMAFLORA, β -HYDROXYETHYLHYDRAZINE

Omaflora, used to control the date of flowering of pineapples, is developed with cinnamaldehyde in acid ethanol solution.²³⁶ The color body is the hydrazone. Although the maximum absorbance is at 390 m μ , the reading is preferably at 420 m μ where the background absorbance is much less. The method will determine microgram quantities.

Procedure—Pineapples. Cut to obtain representative samples in proper proportions of shell flesh and core. Slice about 200 grams into small fragments. Blend with 100 ml. of water. Filter and wash. Convert 50-100 mesh Dowex 1-X8 to the hydroxyl form. Transfer about 80 grams as a slurry to a 40 \times 600-mm. chromatographic column. Wash until neutral. Prepare a 20 \times 400-mm. column with 20 grams of 50-100 mesh Dowex 50W-X8. Wash with 3:7 hydrochloric acid until the washings are colorless, then with water until the washings are neutral.

Pass the filtrate and washings through the hydroxyl-ion resin column, then through the hydrogen-ion column. Wash the latter with water until the washings are neutral. The Omaflora is on the hydrogen-ion column. Elute it with 3:7 hydrochloric acid and dilute to 100 ml. with that acid. Filter to remove cloudiness.

As the color reagent, mix 95 ml. of 95% ethanol, 8.5 ml. of water, and 6 ml. of concentrated hydrochloric acid. When cool, add 2 ml. of *trans*-cinnamaldehyde. Store in a brown bottle and prepare daily.

Mix 6 ml. of the sample with 18 ml. of this reagent. After 30 minutes, read at 420 m μ against a reagent blank. Subtract a blank obtained with untreated pineapple. The color is stable for at least 2 hours. Use fresh resin columns for each determination.

BENZYLHYDRAZINE

Hydrazines such as benzylhydrazine are determined fluorometrically by reaction with 1,2-naphthoquinone-4-sulfonic acid.²³⁷

²³⁶ M. P. Thomas and H. J. Ackermann, *J. Agr. Food Chem.* **12**, 432-3 (1964).

²³⁷ M. Roth and J. Rieder, *Anal. Chim. Acta* **27**, 20-6 (1962).

Procedure—Plasma. Mix 0.1 ml. of plasma containing 1.5% of potassium oxalate and 1.8 ml. of water. Add 0.1 ml. of 20% metaphosphoric acid solution. Shake and then centrifuge for 5 minutes. Adjust 1.5 ml. of the supernatant layer to pH 6.0 by adding 0.9 ml. of 20% solution of trisodium phosphate. Add 0.24 ml. of 0.01% solution of 1,2-naphthoquinone-4-sulfonic acid. Mix and irradiate at 340 $m\mu$ for exactly 8 minutes. Read the fluorescence at 470 $m\mu$.

Organs and tissues. As a buffer for pH 3, dissolve 33.64 grams of citric acid monohydrate and 14.64 grams of disodium phosphate dihydrate in water and dilute to 1 liter. Homogenize 1 part of sample with 2.5 parts of buffer. Mix 1.25 ml. of the sample in the buffer with 1.75 ml. of water. Shake for 15 minutes and centrifuge for 10 minutes. Mix 2 ml. of the upper layer with 0.2 ml. of 2% sodium hydroxide solution. Add 6 ml. of chloroform and 0.75 gram of anhydrous sodium sulfate. Shake for 15 minutes and centrifuge. Remove 4 ml. of the chloroform phase. Shake for 5 minutes with 1 ml. of 1:110 hydrochloric acid and centrifuge. Prepare a buffer for pH 6 of equal volumes of 2.72% monopotassium phosphate and 2.82% anhydrous disodium phosphate solutions. Mix 0.8 ml. of centrifugate, 0.2 ml. of 1.6% sodium hydroxide solution, and 1 ml. of the buffer. Proceed as for plasma, from "Add 0.24 ml. of 0.01% solution. . . ."

HYDROXYLAMINE

Hydroxylamine reacts quantitatively with an excess of 8-quinolinol in the presence of ethanol and sodium carbonate to form 5,8-quinoline-5-(8-hydroxy-5-quinolyimide), also known as indooxine²³⁸ (cf. Vol. IV, p. 55). Beer's law is followed at 705 $m\mu$ over the range of 0 to 5×10^{-2} millimole of hydroxylamine per ml. of solution.

Hydroxylamine reacts with formaldehyde and persulfate to produce formohydroxamic acid, which combines with ferric ion to form a colored salt.²³⁹ Oxalic acid, phosphoric acid, hydrogen fluoride, arsenate, cinnamaldehyde, salicylaldehyde, and vanillin interfere. Tartaric and citric acids deepen the color. Beer's law is followed for 0.02-0.2 mg. of hydroxylamine per ml.

Hydroxylamine is oxidized by iodine to nitrite, which is then de-

²³⁸ D. S. Frear and R. C. Burrell, *Anal. Chem.* **27**, 1664-5 (1955).

²³⁹ Omar A. Guagnini and Eugenio E. Vonesch, *Anales asoc. quim. argentina* **41**, 78-82 (1953).

veloped with sulfanilic acid and 1-naphthylamine.²⁴⁰ Hydroxylamine and β -aspartyl hydroxamate may be determined in one mixture, since hydroxylamine is oxidized at pH 2.3 and 3.7, and hydroxamate is oxidized at pH 3.7 only. Acidification after oxidation provides for a more rapid color development. Beer's law is followed up to 0.1 micromole per compound.

Hydroxylamine may be determined indirectly by oxidation with an excess of potassium chromate and determination of the excess chromate with *o*-dianisidine.²⁴¹ Hydroxylamine may also be read by the color developed with ferric chloride and nitric acid.

Procedure—By 1-quinolinol. To 1 ml. of sample containing up to 0.25 micromole of hydroxylamine, add 1 ml. of 0.05 *M* phosphate buffer at pH 6.8 (Vol. I, p. 174) and dilute to 2.8 ml. with water. Add 0.2 ml. of 12% trichloroacetic acid solution and 1 ml. of 1% 8-quinolinol solution in absolute ethanol. Swirl gently. Add 1 ml. of 11% sodium carbonate solution and shake vigorously. Stopper and heat at 100° for 1 minute to develop the green color. Cool for 15 minutes and read at 705 $m\mu$ against a reagent blank.

By formaldehyde and persulfate. To a solution containing 0.02-0.2 mg. of hydroxylamine per ml., add 1 ml. of 40% formaldehyde solution and 1 ml. of 0.05% ferric alum solution. Dilute to 5 ml. Add 0.1 gram of potassium persulfate, stir, and heat at 35-40° for 15 minutes. Read at 520 $m\mu$.

By iodine. To a 1-ml. sample at 10-15° containing 0.02-0.1 micromole of hydroxylamine, add 5 ml. of 0.1 *M* acetate buffer for pH 3.6, 1 ml. of 1% sulfanilic acid solution in 30% acetic acid, and 0.5 ml. of 1.3% iodine solution in glacial acetic acid. Maintain a temperature of 10-15° for the entire procedure. Shake and let stand for 3-5 minutes. Remove excess iodine by adding 0.5 ml. of 2.5% sodium thiosulfate solution. Add 0.5 ml. of 1:4 hydrochloric acid and stir. Develop the color with 1 ml. of 0.3% 1-naphthylamine solution in 30% acetic acid solution. Dilute to 10 ml. and read at 530 $m\mu$ after 20 minutes.

Indirectly by chromate and o-dianisidine. See hydrazine on page 171.

²⁴⁰ Jacob Yashphe, Yeheskel S. Halpern and Nathan Grossowicz, *Anal. Chem.* 32, 518-20 (1960).

²⁴¹ F. Buscaróns, J. Artigas and C. Rodríguez-Roda, *Anal. Chim. Acta* 23, 214-16 (1960).

By ferric chloride and nitric acid. Mix 10 ml. of sample such as a photographic developer with 2 ml. of 10% sodium hydroxide solution and 0.7 ml. of ethyl acetate. After saponification is complete, add 10 ml. of 1:3 nitric acid. If a green precipitate forms at this point, filter at once. Add 1 ml. of 30% ferric chloride solution and mix. After 5 minutes, dilute to 200 ml. with water. Read after 5 minutes and within 20 minutes.

ARYLHYDROXYLAMINES

Arylhydroxylamines are determined by treatment with ferrocyanide or pentacyanoammineferoate^{11,242} If the sample is in ethanol solution, the sensitivity is increased by extraction of the ethanol with ether, benzene, or chloroform. The methods will detect 1-3 mg. per liter; that with the pentacyanoammineferoate is the more sensitive. Nitrosobenzene interferes with the determination of *N*-phenylhydroxylamine, but other metabolites of aniline do not.

Procedure—By ferrocyanide. To the sample in urine or dilute ethanol, add 0.25% of salicylaldehyde. After 3 hours at 23°, take a 5-ml. test sample. Add 0.1 ml. of 5% potassium ferrocyanide solution. Heat at 70° for 20 minutes and cool in ice water. Read in the range of 490-570 m μ against a reagent blank.

Alternatively, without adding the salicylaldehyde, add 1 ml. of sample to 9 ml. of 1% aqueous potassium ferrocyanide solution. Heat at 60° for 5 minutes, store at 23° overnight, and read as above.

*By pentacyanoammineferoate*¹¹. Add 1 ml. of sample to 5 ml. of 0.05% aqueous pentacyanoammineferoate. Read after 2-3 hours at 490-570 m μ against a reagent blank.

β -ASPARTYL HYDROXAMATE AND OTHER HYDROXAMATES

In the range of 0.02-0.1 micromole per sample, β -aspartyl hydroxamate is determined by oxidation with iodine to nitrite, which is then developed by sulfanilic acid and 1-naphthylamine.²⁴³ The maximal color intensity for β -aspartyl hydroxamate occurs at pH 3.7, with no color development at pH 2-2.3. Similar results are obtained with *succinyl hydroxamic*, γ -

²⁴² E. Boyland and R. Nery, *Analyst* **89**, 95-102 (1964).

²⁴³ Jacob Yarshphe, Yeheskel S. Halpern and Nathan Grossowicz, *Anal. Chem.* **32**, 518-20 (1960).

glutamyl hydroxamic, lacthydroxamic, acetohydroxamic, cinnamohydroxamic, salicylhydroxamic and benzohydroxamic acids.

Procedure—*By iodine.* Follow the procedure under hydroxylamine, substituting 5 ml. of 5% sodium acetate solution for 5 ml. of 0.1 M acetate buffer at pH 3.6.

MILONTIN, *N*-METHYL- α -PHENYLSUCCINIMIDE

Milontin reacts with iodine to form a highly fluorescent product, *N*-methyl- α -phenylmaleimide.²⁴⁴ The urinary metabolites of Milontin do not interfere. Beer's law is followed up to 0.01 mg. per ml.

Sample—*Blood serum or plasma.* Dilute a 1-ml. sample with 3 ml. of water. Shake for 20 minutes with 20 ml. of chloroform. Centrifuge if necessary to completely separate the layers. Develop a 10-ml. portion of the chloroform phase with iodine.

Urine. Neutralize the sample, or make it slightly alkaline with sodium bicarbonate. Follow the procedure for blood serum or plasma.

Tissues. Homogenize a sample with 10 ml. of water. Follow the procedure under blood serum or plasma, starting with "Shake for 20 minutes . . ." using 8 ml. of the homogenate. If the final solution is not neutral or slightly alkaline, add sodium bicarbonate.

Procedure—To prepare a borate buffer, dissolve 37 grams of boric acid and 31 grams of potassium hydroxide in 900 ml. of methanol.

To a 10-ml. chloroform solution containing 0.001-0.01 mg. of Milontin per ml., add 5 ml. of 90% methanol, 1 ml. of borate buffer, and 0.1 ml. of 5% iodine solution in methanol. Mix thoroughly and let stand in the dark at room temperature for 1 hour. Decolorize the excess iodine with 1 drop of 3% hydrogen peroxide. Add 10 ml. of water and shake for 5 minutes. Allow the phases to separate and discard the aqueous phase. Chill in a deep-freeze unit and centrifuge to eliminate any turbidity from remaining water. Warm a portion of the chloroform phase to 25° and determine the fluorescence against a reagent blank. The temperature at which it is read must be accurately controlled.

²⁴⁴ Anthony J. Glazko, Wesley A. Dill, Loretta M. Wolf and Charles A. Miller, *J. Pharmacol. Exptl. Therap.* **111**, 413-24 (1954).

LIDOCAINE, 2-DIETHYLAMINO-2,6-ACETOXYLIDIDE

The analysis of the local anesthetic, lidocaine, is based on the analysis of tertiary amines with *cis*-aconitic anhydride.²⁴⁵ Tertiary amines which are extractable with toluene interfere.

Procedure—To prepare the reagent, add 40 ml. of acetic anhydride to 0.25 gram of *cis*-aconitic anhydride. Swirl to dissolve. Warm gently if necessary. Dilute to 100 ml. with toluene, mix, and allow to age for 24 hours at room temperature before use.

Make the sample alkaline with ammonium hydroxide. Extract with toluene. Centrifuge the toluene layer and dilute so that the sample contains 0.12-0.012 mg. of lidocaine per ml. of toluene. To a 2-ml. aliquot, add 1 ml. of *cis*-aconitic anhydride reagent. Mix, and stopper loosely. Heat at 100° for 45 seconds and let stand at room temperature for 15 minutes. Dilute to 10 ml. with a 1:4 acetic anhydride-toluene mixture to prevent precipitation of the colored complex. Read at 550 m μ after 15 minutes.

MEPROBAMATE, 2-METHYL-2-N-PROPYL-1,3-PROPANEDIOL DICARBAMATE

A general reagent for meprobamate is an aldehyde in the presence of antimony trichloride.²⁴⁶ The aldehyde may be furfural or *p*-dimethylaminobenzaldehyde.²⁴⁷ A probable explanation of the mechanism of the reaction is a furylidene coupling through the amide group of the carbamate in the presence of a dehydrating agent. The color is specific for unsubstituted amides.

With furfural as the aldehyde, Beer's law is followed up to 0.1 mg. per ml. at 570 m μ . With *p*-dimethylaminobenzaldehyde, Beer's law is followed from 0.0005 to 0.01 mg. per ml. at 550 m μ . Extraction procedures permit removal of meprobamate from interferences by urea and naturally occurring amides, and separation from hydroxymeprobamate and the glucuromide conjugate of the drug. As an alternative method of eliminat-

²⁴⁵ Edward G. Feldman and Henry M. Koehler, *J. Am. Pharm. Assoc. Sci. Ed.* **48**, 549-52 (1959).

²⁴⁶ B. J. Ludwig and Allan J. Hoffman, *Arch. Biochem. Biophys.* **72**, 234-5 (1957); G. Lagrange and J. J. Thomas, *J. pharm. Belg.* **13**, 402-8 (1958); Hanichiro Aramaki, *Kagaku to sosa* **12**, 44-9 (1959); O. D. Madsen and V. Schmidt, *Ugeskr. Læge.* **122**, 441, 891-3 (1960).

²⁴⁷ Allan J. Hoffman and B. J. Ludwig, *J. Am. Pharm. Assoc., Sci. Ed.* **48**, 740-2 (1959).

ing interference by urea, add one drop of glycerol-urease extract per ml. to sample, standard, and blank. After incubating at 50° for 15 minutes, cool to room temperature.²⁴⁸

Another determination of meprobamate is based on the determination of the NH group. The meprobamate reacts at room temperature with a hypochlorite solution at pH 10.5 to form an active chlorine derivative, which then reacts with an excess of potassium iodide. The iodine formed is measured as potassium triiodide or as the starch-iodine blue complex.²⁴⁹ When the potassium triiodide color is read, Beer's law is followed up to 0.03 mg. per ml. Urine samples are extracted with ether and separated from interferences by passage through an anhydrous potassium carbonate column.

Meprobamate in chloroform is read directly in the infrared at 6.32 μ .²⁵⁰ Although up to 10 mg. meprobamate per ml. of chloroform can be extracted, the optimum concentration is 4 mg. per ml. Beer's law is followed for 2 to 10 mg. per ml. of chloroform. Stearates do not interfere.

Samples—Plasma or serum. To a 1-ml. sample, add 2 drops of 28% ammonium hydroxide solution, 2 drops of a saturated potassium chloride solution, and 25 ml. of a 1:1 mixture of carbon tetrachloride and chloroform. Shake vigorously for 5 minutes and centrifuge for 10 minutes. Carefully remove the organic layer with a hypodermic syringe and filter through glass wool previously extracted with chloroform. Rinse the glass wool with 5 ml. of the 1:1 mixture of carbon tetrachloride and chloroform. Evaporate the extract and rinsings to dryness at 80°. Rinse down with a few ml. of acetone and heat until thoroughly dry. Develop with *p*-dimethylaminobenzaldehyde and antimony trichloride.

Urine. Dilute the sample 1:5 or 1:10. Follow the procedure for serum or plasma above, ending at "Rinse down with a few ml. of acetone and heat until thoroughly dry." Heat the residue for 15 minutes at 50° with 1 ml. of 1:59 hydrochloric acid. Make the solution alkaline with ammonium hydroxide and repeat the technic for serum or plasma, starting at the beginning.

Urine and serum. Extract a 2-ml. sample with 5 ml. of ether, and evaporate the ether. Treat the residue with 4 ml. of water and 1 ml. of

²⁴⁸ S. J. Kanter, *Clin. Chim. Acta* **8**, 2-4 (1963).

²⁴⁹ Gordon H. Ellis and Charles A. Hetzel, *Anal. Chem.* **31**, 1090-1 (1959).

²⁵⁰ Wm. R. Maynard, *J. Assoc. Offic. Agr. Chemists* **43**, 791-2 (1960).

2% urease suspension in water. Incubate for 45 minutes at 45°. Cool to room temperature and precipitate with 0.5 ml. of a reagent containing 20 grams of phosphotungstic acid and 3 ml. of sulfuric acid per 100 ml. of water. Centrifuge, decant the supernatant liquid, and filter. Rinse the filter paper with 1 ml. of a phosphate buffer at pH 7.4 (Vol. I, p. 174). Shake with 6 ml. of ethyl ether for 5 minutes. Centrifuge and evaporate a 4-ml. portion of the ether layer to dryness. Add 3 ml. of benzene to the residue and evaporate to dryness in an oil bath at 90°. Develop with antimony trichloride and furfural.

Urine. Shake a 4-ml. sample vigorously with 30 ml. of ether for 90 seconds. Let the layers separate for 2-3 minutes and drain off the aqueous phase completely along with a little of the ether. Wash the ether layer twice with 3-4 ml. portions of water, shaking for a few seconds for the second wash. Allow time for complete separation and drain off the water phase with a little of the ether. Run a 15-ml. portion of the ether phase through granular anhydrous potassium carbonate in a 7-mm. × 25-cm. column. As soon as the ether meniscus reaches the top of the column, wash the sample through with 10 ml. of ether. Evaporate the ether at 100° and dissolve the residue in 5-10 ml. of water. Develop by reaction with hypochlorite.

Procedure—By *p*-dimethylaminobenzaldehyde and antimony trichloride. Prepare a saturated solution of antimony trichloride of a concentration of approximately 25% in chloroform by warming to dissolve, cooling, and filtering. Combine four volumes of the antimony trichloride solution with 1 volume of acetic anhydride and store under refrigeration.

To the extracted residue of the sample containing 0.005-0.01 mg. of meprobamate, add 0.2 ml. of a 3:1 mixture of acetone and glacial acetic acid, 0.2 ml. of 1% *p*-dimethylaminobenzaldehyde solution in benzene, and 1 ml. of antimony trichloride-acetic anhydride reagent. Mix, stopper, and heat at 50° for exactly 10 minutes. Cool in water and dilute with 1 ml. of benzene. Read at 550 m μ .

By furfural and antimony trichloride. To an extracted residue of the sample containing up to 0.1 mg. of meprobamate, add 0.1 ml. of glacial acetic acid. Stopper, and heat at 75-80° for 10 minutes. Cool, and add 2 ml. of 20% antimony trichloride solution in chloroform and 0.05 ml. of furfural. Read after 30 minutes at 570 m μ .

By hypochlorite. To prepare a borate buffer for pH 10.5, dissolve 3.1 grams of boric acid and 3.7 grams of potassium chloride in water. Adjust the pH to 10.5 with sodium hydroxide solution and dilute to 1 liter. To prepare the chlorinating solution, dilute 5.25% sodium hypochlorite solution 1:30 with the borate buffer. The potassium chloride in the buffer increases the stability of the chlorinating solution. Alternatively, prepare a chlorinating solution from calcium hypochlorite using a 0.09% solution of calcium hypochlorite containing 74% available chlorine. Dilute 1:30 with the buffer.

To a 1-ml. sample of meprobamate solution add 1 ml. of chlorinating solution. Shake gently to mix and let stand for 15 minutes. Add 1 ml. of 0.5% phenol solution in 1:110 hydrochloric acid to decompose excess hypochlorite. Shake gently. After 5 minutes, add 3 ml. of 0.3% potassium iodide solution and read after 5 minutes at 350 m μ against water.

Directly in the infrared. Weigh 20 tablets or capsules and obtain an average weight per capsule or tablet. Grind and mix. Weigh a portion equivalent to 100 mg. of meprobamate. Dilute to 25 ml. with chloroform and shake frequently over an interval of 10 minutes for all samples except coated tablets, which are shaken for 1 hour. Filter if necessary, discarding the first 10 ml. of filtrate. Read with a double beam infrared spectrophotometer at 6.32 μ , using 0.2 mm. sodium chloride cells with a fixed slit width opening at 150.

GLUCOSAMINE, GALACTOSAMINE AND OTHER HEXOSAMINES

Amino sugars react with acetylacetone in an alkaline medium to form a pyrrole derivative. The latter reacts with *p*-dimethylaminobenzaldehyde to yield a red color read at 530 m μ ²⁵¹ (cf. Vol. IV, p. 57). The method is applicable only to free amino sugars. Large amounts of non-nitrogenous sugars, urea, and glyceraldehyde interfere. Separation of tissue hexosamines from interfering chromogens by ion exchange is not necessary. Impure acetylacetone reagent produces the same color as the sample.²⁵²

²⁵¹ C. J. M. Rondle and W. T. J. Morgan, *Biochem. J.* **61**, 586-9 (1953); B. N. Gladyshev, *Biokhimiya* **21**, 227-30 (1956); L. Bolognani, C. Coppi, and V. Zabotti, *Boll. soc. ital. biol. sper.* **34**, 1950-1 (1958); C. Cessi and Franca Piliego, *Biochem. J.* **77**, 508-10 (1960); J. Svejcar, I. Prerovsky and J. Linhart, *Coll. Czech. Chem. Commun.* **28**, 728-32 (1963); cf. C. H. Pearson, *Biochem. J.* **88**, 540-5 (1963); J. Svejcar, *Chem. Listy* **58**, 570-3 (1964).

²⁵² B. Rosenlund, *Scand. J. Clin. & Lab. Invest.* **8**, 343-4 (1956).

Modification of the procedure by extraction of the reaction product of hexosamine and acetylacetone with isoamyl alcohol minimizes interferences from color from hydrolyzates and nonglucosamine substances that would react with *p*-dimethylaminebenzaldehyde.²⁵³ This modification also eliminates any need for ion exchange separation. Beer's law is followed for 0.008-0.032 mg. of glucosamine or galactosamine per 4 ml. of isoamyl alcohol. Glucose does not interfere.

The reaction product of hexosamine and acetylacetone may be separated from amino acids and glucose by fractional distillation.²⁵⁴ With this modification, methyl amine interferes. Alanine, valine, glycine, and lysine do not interfere. The molar extinction given by hydroxyproline is about 0.3% of that given by glucosamine. Addition of sodium sulfite to the acetylacetone reagent decreases hydroxyproline interference but reduces the sensitivity of the method. Beer's law is followed for 0.005-0.05 mg. of amino sugar.

In the determination of amino sugars in animal, vegetable, and bacterial samples, the sample is fixed and extracted with acetone, water, or an alcoholic solution of formalin to prevent the formation of nitrogenous and carbohydrate condensates. The hydrolyzates of the samples in hydrochloric acid are purified by adding the sample to a corresponding volume of a mixture of trichloroacetic acid, sodium hydroxide, and sodium chloride.²⁵⁵ To separate galactosamine from glucosamine, pass the sample buffered to pH 5.2 through a column of Dowex 50. Then elute the galactosamine with 1:5 hydrochloric acid.²⁵⁶

D-Galactosamine may be determined in the presence of D-glucosamine by condensing the amino sugar with acetylacetone to a pyrrole that retains some of the specific configuration of the sugar, splitting the lateral chain by borate, and reacting the evolved 2-methylpyrrole with *p*-dimethylaminobenzaldehyde.²⁵⁷ Interference by glucosamine is 2%. Beer's law is followed for 0.02-0.1 mg. of D-galactosamine.

Glucosamine and galactosamine may be determined in the presence of each other by conversion to their *N*-acetyl derivatives with acetic anhydride in aqueous solution. When reacted with *p*-dimethylaminobenzaldehyde, these acetylated derivatives yield products that differ in

²⁵³ Otto W. Neuhaus and Marcia Letzring, *Anal. Chem.* **29**, 1230-3 (1957).

²⁵⁴ C. Cessi and France Piliego, *Biochem. J.* **77**, 508-10 (1960).

²⁵⁵ B. N. Gladyshev, *Biokhimiya* **24**, 789-93 (1959).

²⁵⁶ I. R. Johnston, *Biochem. J.* **86**, 254-8 (1963); cf. Masahiko Komoto, *J. Agr. Chem. Soc. Japan* **136**, 310-13 (1962).

²⁵⁷ C. Cessi and Franca Serafini-Cessi, *Biochem. J.* **82**, 43P (1962); **88**, 132-6 (1963).

chromogenic properties.²⁵⁸ The galactosamine must be present in a 17:1 ratio to the glucosamine. The method is subject to interferences from neutral sugars and amino acids and is less sensitive than the method using acetylacetone condensation. Since the color development is influenced by inorganic salts, the concentrations of ions in the standard solution should be the same as the unknown.

Hexosamines in glucoproteins are liberated by hydrolysis with hot 1:3 hydrochloric acid, neutralized with 12% sodium hydroxide solution, and treated with 2% 2,4-pentanedione solution in 5.3% sodium carbonate solution. This forms the heterocyclic oxazole ring with hexosamines. This product reacts with a modified Ehrlich's reagent in which 0.2 gram of *p*-dimethylaminobenzaldehyde is dissolved in 7.5 ml. of methanol and 7.5 ml. of concentrated hydrochloric acid, to form a pink color.²⁵⁹

Hexosamines in biological samples are deaminated with nitrous acid, and the resulting 2,5-anhydrohexoses are reacted with 2,4-dinitrophenylhydrazine to produce a colored compound.²⁶⁰ This method is applicable to 0.01-0.1 mg. of hexosamine. Free carbonyl groups interfere. Hexosamines deaminated with nitrous acid are alternatively developed with phenol-sulfuric acid.²⁶¹ Interferences are removed by passage through an Amberlite XE-64 column.

2-Amino-2-deoxyhexose and 2-deoxygalactose are read at 325 $m\mu$ in concentrated sulfuric acid after heating.²⁶² For the color developed by hexosamines in 79% sulfuric acid, see Volume IIIA, pages 163-8.

Procedure—By *p*-dimethylaminobenzaldehyde. Eluates from starch electrophoresis. To prepare a carbonate buffer at pH 10, dissolve 8 grams of sodium carbonate and 2.1 grams of sodium bicarbonate in 100 ml. of water. To prepare the acetylacetone reagent, add 0.3 ml. of acetylacetone to 10 ml. of buffer. To prepare the developing Ehrlich's reagent for this technic, dissolve 0.4 gram of *p*-dimethylaminobenzaldehyde in 1.5 ml. of concentrated hydrochloric acid and 13.5 ml. of isoamyl alcohol.

Evaporate a 1-ml. sample to dryness at 100°, using a gentle stream of air. Add 1 ml. of 1:3 hydrochloric acid, stopper, and heat at 100° for 4 hours. Concentrate the hydrolyzates to dryness *in vacuo* over solid sodium hydroxide. Add 0.5 ml. of 0.4% sodium hydroxide solution, 1 drop

²⁵⁸ Saul Roseman and Irmgard Daffner, *Anal. Chem.* **28**, 1743-6 (1956).

²⁵⁹ E. J. Garcia Canturri, *Med. y Seguridad Trabajo* **9**, No. 33, 13-18 (1961).

²⁶⁰ J. Popowicz, *Rozniki akad. Med. im. Juliana Marchlewskiego w Białymstoku* **5**, 319-43 (1959).

²⁶¹ Yun C. Lee and Rex Montgomery, *Arch. Biochem. Biophys.* **93**, 292-6 (1961).

²⁶² J. D. Ciperia, *Analyst* **85**, 517-19 (1960).

of phenolphthalein indicator, and enough 4% sodium hydroxide solution to make the mixture distinctly alkaline. Add 1:110 hydrochloric acid until the pink color just disappears. Dilute to 1 ml. Add 1 ml. of acetyl-acetone solution, mix, and stopper. Heat for 20 minutes at 100°. Cool and shake vigorously with 5 ml. of isoamyl alcohol for 2 minutes. Separate the layers by brief centrifuging.

To a 4-ml. aliquot of the alcoholic extract containing 0.008-0.32 mg. of hexosamine, add 1 ml. of *p*-dimethylaminobenzaldehyde reagent. Mix, and read after 15 minutes at 530 $m\mu$ against a reagent blank.

Biological materials. Cover the sample completely with acetone. Refrigerate for 2 days, changing the acetone twice. Vacuum dry and extract 4 times with 40 volumes of a boiling 5:1 mixture of formaldehyde and 95% ethanol. If the sample contains large quantities of lipids, such as certain plant seeds and egg yolks, extract first with ether. Vacuum dry and bring to constant weight at 50°.

Reflux a 100-250-mg. sample for 6 hours with 5-10 ml. of 1:1 hydrochloric acid. Decant or filter the hydrolyzate and neutralize with sodium carbonate or sodium bicarbonate. Saturate the hydrolyzate filtrate with sodium chloride.

Take a 3-4 ml. portion. For each ml. of sample, add 1.25 ml. of a 1:1 mixture of concentrated hydrochloric acid and 80% trichloroacetic acid solution. A fatty-appearing acetone-insoluble brown liquid consisting of a mixture of the products of hydrolysis and the sodium trichloroacetate will settle to the bottom. Add sodium carbonate to the settled mixture to remove some color-producing substances and improve the optical properties of the products of hydrolysis. This step should proceed to the point of complete neutralization of the excess hydrochloric acid and should be performed carefully to avoid redissolving the settled impurities. Filter.

Neutralize 1 ml. of the hydrolyzate or 0.1-0.5 ml. diluted to 1 ml. with water. Add 1 ml. of 2% acetyl-acetone solution in 8% sodium carbonate solution. Rinse the walls of the tube with 1 ml. of water. Stopper, and heat at 100° for 10 minutes. Cool rapidly, add up to 8 ml. of a *p*-dimethylaminobenzaldehyde reagent prepared by adding 0.8 gram to 30 ml. of concentrated hydrochloric acid and 30 ml. of 95% ethanol. Dilute to 10 ml. with 95% ethanol and read at 530 $m\mu$ after 40 minutes.

As a pyrrole derivative with p-dimethylaminobenzaldehyde. For the acetylacetone reagent, dissolve 1 ml. of redistilled acetylacetone in 50 ml. of 8% sodium carbonate solution. The reagent is stable for 2-3 hours at

18°. Reprecipitate purified *p*-dimethylaminobenzaldehyde from 95% ethanol by adding 50-75% of water. Dissolve 0.8 gram in 30 ml. of 95% ethanol and add 30 ml. of concentrated hydrochloric acid. Store at -10°.

Take a 1-ml. sample containing up to 0.3 mg. of glucosamine. If the solution is acid, add 1 drop of phenolphthalein indicator solution and enough 4% sodium hydroxide solution to give a full pink color. Add 1:39 hydrochloric acid dropwise until the pink color just disappears. Wash the walls of the tube with water and dilute to 3 ml. Add 1 ml. of acetylacetone reagent and mix by shaking gently. Wash down the walls of the tube with 1 ml. of water. Close the tube with a 2-3-cm. long-necked sealed glass ampule, which acts as a condenser to prevent loss of acetylacetone. Heat at 100° for 20 minutes. Cool, and add 5 ml. of 95% ethanol. Add 1 ml. of *p*-dimethylaminobenzaldehyde reagent and dilute to 10 ml. with 95% ethanol. Mix gently, and heat at 65-70° for 10 minutes to liberate carbon dioxide. Cool to 18°, mix, and read at 530 $m\mu$.

Alternatively, prepare a sodium carbonate-sodium bicarbonate buffer by dissolving 23.02 grams of sodium carbonate, 2.76 grams of sodium bicarbonate, and 5.84 grams of sodium chloride in 1 liter of water. The pH should be 9.8. As a reagent, dissolve 1 ml. of colorless, redistilled acetylacetone in 100 ml. of the buffer. As *p*-dimethylaminobenzaldehyde reagent, dissolve 80 mg. in 100 ml. of absolute ethanol containing 3.5 ml. of concentrated hydrochloric acid.

Mix a 2-ml. sample containing 0.005-0.05 mg. of amino sugar or mixtures of amino acids and glucose with 5.5 ml. of acetylacetone reagent. Heat at 100° for 20 minutes. Cool in water. Transfer to a distillation apparatus and wash the reaction tube with three 2-ml. portions of water, adding to the solution. Distil and collect 2-ml. portions in 8 ml. of *p*-dimethylaminobenzaldehyde reagent. Read at 545 $m\mu$ after 30 minutes.

D-Galactosamine in the presence of D-glucosamine. To prepare the borate buffer for pH 8, dissolve 50 grams of potassium tetraborate and 40.6 grams of boric acid in 1 liter of water. Dry a 0.1-0.5-ml. sample containing 0.02-0.1 mg. of amino sugar hydrochloride at 50° *in vacuo* in a stoppered tube. Add 0.5 ml. of absolute methanol containing 20% of triethylamine, 6% of acetylacetone, and 1% of pyridine. Incubate for 16 hours at 55°. Remove the reagent *in vacuo* and heat the stoppered tube for 20 minutes with 6 ml. of borate buffer at 100°. Cool to 35° and distil 2 ml. Collect in 8 ml. of a reagent containing 0.8% of *p*-dimethylaminobenzaldehyde and 3.5% of concentrated hydrochloric acid. Read at 550 $m\mu$.

As *n*-acetyl derivatives with *p*-dimethylaminobenzaldehyde. *Hexosamine-containing polymers.* Prepare the acetic anhydride reagent prior mixture 4 *N* with respect to acid. Seal and fully immerse in boiling water. To prepare the *p*-dimethylaminobenzaldehyde reagent, dissolve 0.36 gram in 20 ml. of glacial acetic acid. Add 0.5 ml. of concentrated hydrochloric acid and dilute to 50 ml. with glacial acetic acid.

Add concentrated hydrochloric acid to the sample to make the final mixture 4*N* with respect to acid. Seal and fully immerse in boiling water for 5 hours. Cool. Open, and dry in a vacuum desiccator over calcium chloride and soda lime. Evacuate the desiccator with an oil pump to 10^{-3} mm., or lower, of mercury to remove hydrochloric acid. Dissolve the dried residue in water.

To a 1-ml. aliquot of the sample in an acetylation tube, add 0.1 ml. of acetic anhydride reagent and 0.1 ml. of a saturated sodium bicarbonate solution. Mix, and let stand at room temperature for 10 minutes. Destroy the excess anhydride by heating at 100° for 3 minutes. Cool, add 0.5 ml. of 5.3% sodium carbonate solution, and mix vigorously. Heat at 100° for 3 minutes. Cool immediately and dilute to 2 ml. with water. Stopper, and invert several times.

To a 1-ml. aliquot, add 5 ml. of glacial acetic acid with shaking. Add to each of the tubes being run 5 ml. of *p*-dimethylaminobenzaldehyde reagent at 30-second intervals, timed with a stop watch. Shake the tubes vigorously during addition of the reagent. Read at 530 $m\mu$ after 30 minutes from the time of addition of reagent.

Total hexosamine content. To prepare the Dowex 50 ion-exchange resin, wash 250-500 mesh Dowex 50 alternately and slowly several times with 8% sodium hydroxide solution, 1:5 hydrochloric acid, and water.²⁶³ Remove excess water with suction. Prepare a 1:1 dispersion of the resin in water and shake vigorously. Prepare an ion-exchange column from a 10 × 75-mm. borosilicate glass tube, which is pulled out at the tip. Add glass wool to the tip and add 1 ml. of resin suspension containing 0.5 ml. of resin. Pack the glass wool so that the flow rate is 0.1 ml. per minute, using slight air pressure, if necessary, to force the solution through. Follow the procedure for hexosamine-containing polymers, starting with "Add concentrated hydrochloric acid . . ." and ending with "Evacuate the desiccator with an oil pump to remove hydrochloric acid."

Dissolve the dried residue after hydrolysis in 1 ml. of water and

²⁶³ N. F. Boas, *J. Biol. Chem.* **204**, 553 (1953).

transfer to the ion-exchange column. After the liquid has drained to the surface of the resin, wash the hydrolysis tube with three to four 2-ml. portions of water and add to the ion-exchange column, allowing each wash to completely drain through the resin before adding the next wash. Elute with 1:24 hydrochloric acid added in 1-ml. portions until 5 ml. is collected.

For the acetic anhydride procedure, dry an aliquot of the eluate *in vacuo* and dissolve in 1.5 ml. of water. Develop a portion containing 0.025-0.35 mg. of glucosamine and 0.1-1.4 mg. of galactosamine, starting at "To a 1-ml. aliquot of the sample in an acetylation tube . . ."

$$\text{Glucosamine} = (A_{\text{mixture}} - (Y_1 + Y_2) - B_2H)/(B_1 - B_2)$$

in which H is the total hexamine concentration; A_{mixture} is the absorbancy of the mixture; B_1 is the slope of standard curve of glucosamine; B_2 is the slope of standard curve of galactosamine; and Y_1 and Y_2 are intercepts of standard curves on the absorbance axis.

By phenol-sulfuric acid. Treat Amberlite XE-64 successively with 4% sodium hydroxide solution and 1:10 hydrochloric acid and decant. Wash the final resin in the H^+ form with water until it is free from chloride. Dry at room temperature in a stream of air. The ion-exchange apparatus consists of tubing 15-20 cm. long and 6 mm. in internal diameter, tapered to about 1 mm. Pack the constricted end with fine glass wool so that 5 ml. of water will drain in 10-15 minutes. Suspend 100 mg. of dry Amberlite XE-64 H^+ form in 5 ml. of water for 10-15 minutes. Transfer to the ion-exchange tube and treat with 2 ml. of 4% sodium hydroxide solution, 2 ml. of water, 5 ml. of 14% sodium phosphate buffer at pH 6.8 (Vol. I, p. 174) and 5 ml. of water.

Add a sample solution containing 0.08-0.5 mg. of hexosamine hydrochloride to the column. Add 5 ml. of water to wash out reducing sugars. Elute the hexosamines with 0.5 ml. of 1:11 sulfuric acid and 3.5 ml. of water added in one 0.5-ml. portion and three 1-ml. portions. Collect the 4 ml. of effluent and add 8% sodium hydroxide solution dropwise until the solution is just alkaline, or yellow to 0.05% *p*-nitrophenol indicator.

Immediately add 30% acetic acid until the reaction mixture is just colorless. Dilute to 5 ml. with water. To 1.5 ml. aliquots containing 0.025-0.12 mg. of hexosamine, add 0.2 ml. of 5% sodium nitrite solution and 0.2 ml. of 40% acetic acid. Shake to mix. After 10 minutes at room temperature, add 0.2 ml. of 15% ammonium sulfamate solution. Shake vigorously for 30-40 seconds and let stand for 30 minutes, shaking every

10 minutes. Add 0.1 ml. of 80% phenol solution and 5 ml. of concentrated sulfuric acid and read at 490 $m\mu$ after 20-30 minutes.

Blood serum. Heat a sample of 0.05-1 ml. with 0.5 ml. of 1:2 hydrochloric acid for 4 hours at 105° to hydrolyze it. Neutralize with 0.5 ml. of 8% sodium hydroxide solution. As an acetylacetone reagent, prepare a buffer of 0.276% of sodium bicarbonate, 0.584% of sodium chloride, and 2.302% of sodium carbonate. Dissolve 1% of acetylacetone in this and add 5 ml. to the hydrolysate. Heat at 100° for 20 minutes and cool. As a development reagent, prepare at 2% solution of *p*-dimethylaminobenzaldehyde in 1:1 hydrochloric acid. Transfer 3 ml. of sample with 3 ml. and 3 ml. of the buffer. Distil into 4 ml. of the freshly diluted *p*-dimethylaminobenzaldehyde reagent. Read at 535 $m\mu$ after 30 minutes.

*By ninhydrin. Protein.*²⁶⁴ Prepare a 0.9 × 30-cm. column of Amberlite CG 120 of 40 ± 7 microns. Treat it with 10.3% sodium citrate solution adjusted to pH 5.26. Hydrolyze a sample containing 0.2 mg. of hexosamines in 1 ml. of 1:2 hydrochloric acid in a sealed tube at 100° for 16 hours. Apply 0.2 ml. of the acid hydrolysate to the column. Elute with the buffer for pH 5.26 at 20 ml. per hour, collecting 1 ml. fractions. Glucosamine appears in fractions 25-27 and galactosamine in fractions 27-30. Develop with ninhydrin.

Direct reading. To 1 ml. of aqueous solution, add 7 ml. of concentrated sulfuric acid. Heat for 10 minutes and read at 325 $m\mu$. There are other maxima at 248, 283, 289, and 298 $m\mu$.

N-ACETYLAMINO SUGARS

N-Acetylglucosamine and *N*-acetylgalactosamine are measured by reaction with *p*-dimethylaminobenzaldehyde.²⁶⁵ A possible mechanism for the reaction involves the intermediate formation of a glucosazoline when the acetylamine sugar is heated with alkali, followed by reaction of the intermediate with the benzaldehyde in acid. The reaction is carried out in a borate buffer to eliminate interference from changes in pH. The optimum pH is 8.9. The color may be read at 544 or 585 $m\mu$. At 585 $m\mu$, there is an increase in sensitivity and less interference from sugars plus amino acids.

²⁶⁴ S. M. Partridge and D. F. Elsdon, *Biochem. J.* **80**, 34p (1961).

²⁶⁵ Jose L. Reissig, Jack L. Strominger, *J. Biol. Chem.* **217**, 959-66 (1955).

N-Acetylglucosamine-6-phosphate gives the same extinction coefficient as N-acetylglucosamine at 544 and 585 m μ . Uridine diphosphate N-acetylglucosamine, uridine diphosphate N-acetylgalactosamine, and N-acetylglucosamine-1-phosphate do not react unless previously hydrolyzed with acid. Magnesium ions in the range of 0.003 M inhibit the color by 2%. Beer's law is followed up to 0.13 micromole for acetylglucosamine and up to 0.4 micromole for acetylgalactosamine. As little as 3×10^{-10} mole of acetylglucosamine can be determined. For determination of N-acetyl-D-glucosamine by the color in 79% sulfuric acid, see Volume IIIA, pages 164-8.

Procedure—To prepare the potassium tetraborate buffer, adjust a 26% solution of potassium tetraborate to pH 9.1 with 20% potassium hydroxide solution. When diluted 6 times, this buffer should be at pH 8.9. To prepare the reagent, dissolve 10 grams of *p*-dimethylaminobenzaldehyde in 100 ml. of glacial acetic acid containing 12.5% of concentrated hydrochloric acid by volume. Before use, dilute the reagent with 9 volumes of glacial acetic acid.

To a 0.5-ml. sample, blank, and standard, add 0.1 ml. of potassium tetraborate buffer. Heat at 100° for 3 minutes and cool in water. Add 3 ml. of the *p*-dimethylaminobenzaldehyde reagent. Mix and heat at 36-38° for 20 minutes. Cool, and read immediately at 544 or 585 m μ .

N-ACETYLNEURAMINIC ACID

Protein-bound N-acetylneuraminic acid is developed with orcinol.²⁶⁶ For purification, the acid is precipitated from the spinal fluid sample in ethanol.

Procedure—*Spinal fluid*. To prepare the orcinol reagent, mix 100 mg. of orcinol and 40.7 ml. of concentrated hydrochloric acid. Add 1 ml. of 1% ferric chloride solution as an oxidizing agent and dilute to 50 ml. with water.

To 10 ml. of 95% ethanol, add a 2-ml. sample dropwise. Cover and refrigerate for 16 hours to allow complete precipitation of the protein-bound N-acetylneuraminic acid. Centrifuge for 10 minutes. Decant the supernatant liquid and wash the precipitate with 5 ml. of 95% ethanol. Dissolve the precipitate in 3 ml. of water and add 3 ml. of orcinol reagent.

* N. M. Papadopoulos, Sister Mary William and W. C. Hess, *Georgetown Med. Bull.* **14**, 15-8 (1960).

Mix and heat at 100° for 15 minutes. Chill in ice for 5 minutes and extract with 5 ml. of isoamyl alcohol. Centrifuge at 2000 rpm. for 1 minute and read the alcohol layer at 570 m μ against a reagent blank.

QUATERNARY AMMONIUM COMPOUNDS

Quaternary ammonium compounds, except those of low molecular weight, including tetramethylammonium and choline salts are determined by extraction from bromothymol blue solution with chloroform²⁶⁷ (Vol. IV, pp. 66-7). The complex ratio of bromothymol blue molecule to quaternary ammonium compound for the following compounds is: *gal-lamine* 3:1; (*p*-nitrophenoxymethyl)benzyltrimethylammonium chloride 1:1; *hexamethonium* 1:1; *centrimide* 1:2. Up to 5 mg. of sodium sulfite, 0.06 mg. of chlorbutanol, 0.1 gram of dextrose, and 0.1 gram of lactose per 5 ml. do not interfere. Inorganic salts increase the amount of both free indicator and complex extracted, the effect on the reagent blank being greatest at low pH values. Anionic surface-active agents, proteins, and peptones that react with quaternary ammonium compounds interfere. Albumin and polyvinylpyrrolidone may react with the bromothymol blue. However, up to 2.7 mg. of polypyrrolidone does not interfere.

Tetrapropylammonium iodide or any compound of larger molecular weight is determinable with methyl orange.²⁶⁸ The solution is buffered at pH 10.2 and the reagent added. The complex formed is extracted by chloroform. Then the complex is broken by re-extracting the chloroform layer with 1:10 hydrochloric acid. Alkaloids and primary, secondary, and tertiary amines do not interfere.

A similar type of reaction is carried out with Orange II.²⁶⁹ An alternative is precipitation of cationic aliphatic surfactants with excess of potassium 2-bromo-5-chlorobenzenesulfonate. The precipitate is filtered and the excess of reagent read at 288.7 m μ .²⁷⁰

For determination of organic bases in general, 0.1-1 mg. is reacted with ethanolic Erichrome Black T at pH 4-6. The complex formed is extracted into chloroform and read at 510-520 m μ .²⁷¹ Benzethonium

²⁶⁷ C. W. Ballard, J. Isaacs and P. G. W. Scott, *J. Pharm. and Pharmacol.* **6**, 971-85 (1954).

²⁶⁸ R. S. Santoro, *J. Am. Pharm. Assoc., Sci. Ed.* **49**, 666-8 (1960).

²⁶⁹ Masanobu Kato, Yasuji Izawa, Yoshiro Ogata and Wasaburo Kimura, *J. Chem. Soc. Japan, Ind. Chem. Sect.* **66**, 1449-51 (1963).

²⁷⁰ Yasuji Izawa, Takashi Aiki and Wasaburo Kimura, *ibid.* **66**, 1679-82 (1963).

²⁷¹ F. Pellerin, J.-A. Gautier, O. Barat and D. Demay, *Chim. Anal.* **45**, 395-8 (1963).

chloride is determined as the complex with bromophenol blue or methyl orange by extraction with an organic solvent.²⁷²

The specific detectable minimum of some specific organic bases with eosin as the dye is as follows:²⁷³ Bigumal, Chloroguanidine hydrochloride, 29 gamma; Tiphen, *S*-[2-(diethylamino)ethyl] ester of diphenylthioacetic acid, 65 gamma; Spasmolytine, 2-(diethylamino)ethyl ester of diphenylthioacetic acid, 69 gamma; Dimedrol, 2-(diphenylmethoxy)-*N,N*-dimethylethylamine, 60 gamma; Diprophen, *S*-[2-(dipropylamino)-ethyl] ethyl ester of diphenylthioacetic acid, 81 gamma. The reaction, accurate to 10%, is appropriate for control purposes.

Long chain quaternary ammonium compounds in the range of 0.01-0.5 mg. are concentrated on partially carboxymethylated cellulose to permit determination in the presence of fatty materials, especially stearates.²⁷⁴ They are removed quantitatively with ethanolic acid and read as the bromophenol blue-quaternary ammonium complex. Treatment of test tubes with polymetaacrylic acid ester will greatly diminish loss of quaternary ammonium compounds by adsorption when determined by the indicator, bromocresol purple.²⁷⁵ Up to 1% of calcium, magnesium, ferrous, ferric, and cupric ions do not interfere. Beer's law is followed up to 0.025 mg. per ml.

In a strongly alkaline medium, hexanitrodiphenylamine forms complexes with quaternary ammonium compounds. The complexes formed are extracted with chloroform or methylene chloride at pH 11 or with methylene chloride from 12% sodium hydroxide solution.²⁷⁶ The extraction from sodium hydroxide solution is necessary for compounds containing several quaternary ammonium groups. Tetrabutylammonium iodide acts as a stabilizing agent to prevent precipitation. In solutions without tetrabutylammonium iodide, filtration decreases the extinction at 420 m μ . An applicable method by chloranil appears under tertiary aliphatic amines, on page 142. A micro method for cholines by iodine, given on page 200, is applicable to quaternary ammonium compounds.

²⁷² J. Birner, *Australian J. Sci.* **25**, 416 (1963).

²⁷³ V. N. Bernshtein and I. V. Chuiko, *Uch. Zap. Pyatigorskii Gor. Farmatsert. Inst.* **5**, 193-8 (1961).

²⁷⁴ L. D. Metcalfe, *Anal. Chem.* **32**, 70-2 (1960).

²⁷⁵ Jørgen Fogh, Paul O. H. Rasmussen and Knud Skadhauge, *ibid.* **26**, 392-5 (1954).

²⁷⁶ G. Schill, *Anal. Chim. Acta* **21**, 342-52 (1959); G. Schill and B. Danielsson, *ibid.* **21**, 248-54 (1959); G. Schill and M. Marsh, *Svensk. Farm. Tidskr.* **67**, 385-400 (1963).

Determination as the reineckate dissolved in acetone, as given on page 201, is generally applicable to quaternary compounds.

Another reaction is that with dithizone. The product is extracted with chloroform and read at 490 $m\mu$.²⁷⁷ The method is applicable up to 0.01 mg. of the quaternary. Addition of potassium cyanide prevents interference by heavy metals.

Quaternary ammonium compounds in polysaccharides are determined after hydrolysis of the latter with phosphoric acid. The quaternary ammonium cations are quantitatively precipitated by picric acid. The precipitate is extracted by chloroform to read at 365 $m\mu$.²⁷⁸ Unreacted picric acid remains in the aqueous solution. Amino acids or amino sugars do not interfere. The work was done with cetyltrimethylammonium chloride. Poldine methyl methosulfate is a quaternary compound covered in Chapter 8, Volume IVB.

Procedure—By bromothymol blue. To prepare the reagent, dissolve 0.15 gram of bromothymol blue and 0.15 gram of sodium carbonate in 100 ml. of water. To prepare the boric acid buffer for pH 7.5, dissolve 5 grams of boric acid in absolute ethanol, add 20 ml. of water, and dilute with absolute ethanol to 250 ml.

Add 5 ml. of a solution containing up to 2 micromoles of univalent quaternary salt, 1 ml. of bromothymol blue solution, and 20 ml. of buffer to 20 ml. of chloroform. Shake 2 minutes and let separate. Run the chloroform layer into a flask containing 0.4 gram of glass wool. Repeat the extraction and separation with two 20-ml. portions of chloroform. Shake the combined chloroform extracts for 1 minute, let stand for 5 minutes and decant through glass wool into 25 ml. of the boric acid buffer. Wash the flask and filter with successive small amounts of dichloroethane or ethanol-free chloroform. Add the washings to the filtrate and dilute to 100 ml. Read at 420 $m\mu$ against chloroform.

By bromocresol purple. Cetylpyridinium chloride in colorless liquids. Coat glassware with 1-2% Plexiglas solution in chloroform by filling with the solution and draining immediately. Dissolve 0.2 gram of bromocresol purple in a few ml. of 4% sodium hydroxide solution and a small amount of water. Dilute to 200 ml. and adjust the pH to 8.2. Dilute to 300 ml. with water.

²⁷⁷ H. Deppeler and A. Becker, *Z. anal. Chem.* **199**, 414-19 (1964).

²⁷⁸ J. L. Sloneker, J. B. Mooberry, P. R. Schmidt, J. E. Putsley, P. R. Watson and Allene James, *Anal. Chem.* **37**, 243-6 (1965).

Mix a 4-ml. sample containing 0.025 mg. of cetylpyridinium chloride per ml. with 0.1 of bromocresol purple solution and 0.2 ml. of 0.5 *N* disodium phosphate buffer for pH 8.2. Read at 620 $m\mu$ against a reagent blank.

In colored liquids. Prepare 2 mixtures each containing 0.1 ml. of bromocresol purple reagent and 4 ml. of sample solution containing about 0.025 mg. of cetylpyridinium chloride per ml. To 1 mixture, add 0.2 ml. of 0.5 *N* disodium phosphate buffer for pH 8.2. To the other mixture, add 0.2 ml. of buffer consisting of 0.5 *N* disodium phosphate and 2% of anionic detergent, Teepol 410. Read the first mixture against the second at 620 $m\mu$.

By bromophenol blue in the presence of fatty materials. The ion-exchange column consists of a 2.5×40 -cm. glass column packed to a height of 8 cm. with partially carboxymethylated cellulose. Wash the cellulose with 5 ml. of 1:10 hydrochloric acid and wash with water until the washings are neutral. To prepare the ethanolic hydrochloric acid, add 85 ml. of concentrated hydrochloric acid to 1 liter of ethanol.

Add an aliquot of the sample containing 0.1-0.5 mg. of quaternary ammonium compound to the column. Wash with 25 ml. of ethanol and 25 ml. of water. Discard these washings. Add 5 ml. of ethanolic hydrochloric acid to the column and wash with 30 ml. of water. Collect this for development. Add 50 ml. of chloroform and 1 ml. of 0.1% bromophenol blue solution in ethanol. For 0.05-0.1 mg. of quaternary ammonium compound, use 25 ml. of chloroform; for 0.01-0.05 mg., use 10 ml. of chloroform.

Shake for 1 minute, allow the layers to separate, and add the lower chloroform layer to 10 ml. of 1% sodium carbonate solution. Shake for 15 seconds and allow the layers to separate. In the presence of quaternary ammonium compounds, the chloroform layer is blue. Add the chloroform layer to 0.5 gram of anhydrous sodium sulfate and let stand for 30 minutes. Read at 603 $m\mu$ against chloroform.

By methyl orange. Benzethonium chloride. To a 5-ml. sample containing 0.005-0.025 mg. of benzethonium chloride, add 1 ml. of saturated potassium acid phthalate solution, 1 ml. of 0.05% methyl orange solution, and 10 ml. of chloroform. Shake for 1 minute and read the chloroform layer at 420 $m\mu$ 30 minutes later.

By hexanitrodiphenylamine. Monoquaternary compounds. To prepare the reagent, dissolve 10 mg. of hexanitrodiphenylamine in 100 ml. of chloroform. Dissolve $3 \cdot 10 \times 10^{-7}$ equivalents of quaternary ammonium compound in water and dilute to 5 ml. Add 0.3 ml. of 0.4% sodium hydroxide solution, 5 ml. of chloroform, and 5 ml. of hexanitrodiphenylamine reagent. Shake for 15 seconds and separate the organic phase. Repeat the extraction with 5-ml. portions of chloroform until the organic phase remains colorless. Combine the chloroform extracts and dilute to 50 ml. with chloroform. Add 5 mg. of tetrabutyl ammonium iodide as a stabilizer and read at 420 m μ .

Compounds containing several quaternary ammonium groups. As a reagent, dissolve 10 mg. of hexanitrodiphenylamine in 100 ml. of methylene chloride.

Dissolve $3 \cdot 10 \times 10^{-7}$ equivalents of quaternary ammonium compound in water and dilute to 50 ml. Add 20 ml. of methylene chloride, 5 ml. of reagent, and 2 ml. of 40% sodium hydroxide solution. Shake for 10 seconds and draw off the methylene chloride phase. Shake the aqueous phase with 5 ml. of methylene chloride for 1 minute. Add 1 ml. of reagent and shake for 10 seconds. Draw off the organic phase and repeat the extraction until the organic phase is colorless. Dilute the combined methylene chloride fractions to 50 ml. with methylene chloride. Add 5 mg. of tetrabutylammonium iodide and read at 420 m μ .

By picric acid. In polysaccharides. Dissolve a sample of up to 80 mg. in 1.5 ml. of water. Add 1 ml. of 1:5 dilution of 85% phosphoric acid. To destroy the viscosity of the polysaccharide, autoclave at 120° for 20-25 minutes. Cool, and add 1 ml. of 12% sodium hydroxide solution, which has been checked out as exactly neutralizing 1 ml. of the 1:5 phosphoric acid to pH 4.3. Add 0.1 ml. of half-saturated aqueous picric acid, and mix. Extract the precipitate formed, using 1.5, 1.5, and 1.5 ml. of chloroform. Dilute the clear chloroform extracts to 10 ml. and read at 365 m μ .

BENZALKONIUM CHLORIDE

Benzalkonium chloride can be determined by cobaltous chloride and ammonium thiocyanate²⁷⁹ or by stannous chloride.²⁸⁰ With the cobaltous chloride reagent, other quaternary ammonium salts and some secondary

²⁷⁹ Dai Aoki, Yoji Iwayama and Kaoru Oka, *Yakuzaigaku* **16**, No. 1, 11-12 (1956).

²⁸⁰ Kiyoshi Yoshimura and Minoru Morita, *Pharm. Bull.* **3**, 432-4 (1955).

and tertiary amines interfere, while alcohols, glycols, and common solvents do not.

Procedure—*By cobaltous chloride and ammonium thiocyanate.* Shake a 5-ml. sample containing 0.05-0.5% of benzalkonium chloride for 1 minute with 1 ml. of 5.3% sodium carbonate solution and 5 ml. of chloroform. Shake for 1 minute 4 ml. of the chloroform layer with a solution containing 24 parts of cobaltous chloride hexahydrate, 35 parts of ammonium thiocyanate, and 100 parts of water. Filter and read.

By stannous chloride. To a 5-ml. sample containing up to 0.2 mg. of benzalkonium chloride per ml., add 5 ml. of 1:8 sulfuric acid. Cool in ice and add 5 ml. of 2% phosphotungstic acid solution. Shake, and let stand. Filter. Take up the precipitate in 15 ml. of warm acetone. Add 5 ml. of 1% stannous chloride solution in 1:3 hydrochloric acid and 10 ml. of 1:3 hydrochloric acid. Dilute to 50 ml. with acetone. Let stand for 80 minutes at 25° and read at 730 m μ .

DODECYLTRIMETHYLAMMONIUM 2,4,5-TRICHLOROPHENOXIDE

By steam distillation, the test substance is converted to 2,4,5-trichlorophenol. This is then developed with 4-aminophenazone.²⁸¹

Procedure—Grind a sample containing 0.1-0.5 mg. of test substance with 300 ml. of water and 10 ml. of 40% phosphoric acid. Distil with steam into 4 ml. of 4% sodium hydroxide solution. Add 4 ml. of 4% sodium hydroxide solution and 40 ml. of ether to 120 ml. of distillate. Shake to remove interferences and discard the ether layer. Adjust the aqueous layer to pH 7.8 with 10% phosphoric acid.

As a buffer, adjust 1.36% monopotassium phosphate solution to pH 7.8 by addition of 4% sodium hydroxide solution. Add 30 ml. of this buffer, 2 ml. of 1% solution of 4-aminophenazone, and 1.2 ml. of 4% solution of potassium ferricyanide. Dilute to 100 ml. with water and read at 510 m μ after 5 minutes.

Samples under 0.1 mg. Reduce the 4-aminophenazone to 0.5 ml. and the ferricyanide to 0.3 ml. After 5 minutes, shake the 100-ml. dilution with 20 ml. of chloroform. Read the chloroform layer at 480 m μ .

²⁸¹ Tatsu Konda and Iwao Kawashiro, *J. Food Hyg. Soc., Japan* 5, 372-8 (1964).

TRIMETHYLETHANOL AMMONIUM HYDROXIDE, CHOLINE

Choline is precipitated as choline periodide, which is dissolved in ethylene dichloride and read at 365 m μ .²⁸² Reproducibility of this method depends on the accurate aspiration of the supernatant liquid after the centrifuging of the choline periodide precipitate. To reduce the blank, the choline periodide is shaken with ethylene dichloride and water to reduce the effect of any remaining reagent.²⁸³ Interfering triiodide ions remain in the water. With this modification, Beer's law is followed for 0.005-0.75 mg. of choline. The error increases with samples containing less than 0.01 mg. choline. The method is adaptable to micro determination.²⁸⁴

Centrifuging is eliminated entirely by addition of choline to an iodide solution.²⁸⁵ This leads to an increase in total optical density at 365 m μ of the ethylene-dichloride-soluble material. The increase in optical density over the blank value is proportional to the amount of choline present up to 0.05 mg. The following do not interfere in amounts of 0.1 mg.: DL-alanine, L-arginine, L-aspartic acid, L-cysteine, L-cystine, glycine, L-histidine, DL-isoleucine, DL-leucine, DL-lysine, DL-methionine, L-phenylalanine, L-proline, DL-serine, DL-threonine, L-tyrosine, DL-valine, ethanolamine, histamine, D-glucosamine, diphenylamine, adenosine, L-asparagine, L-glutamine and glycyl glycine. Acetylcholine, acetyl β -methylcholine and tryptophan yield precipitates with iodine.

Phosphomolybdic acid precipitates choline to yield a blue color.²⁸⁶ Both the precipitate and reagent are stable. Serine and ethanolamine do not interfere in quantities less than 20 micromoles per 0.5 ml. Up to 16 mg. of kephalin can be tolerated. Ammonia interferes. Reducing sugars such as glucose do not interfere with the reagent.

Dinitrophenylethanolamine and dinitrophenylserine lipids under the conditions of the method form molybdenum blue upon addition of the reagent. The procedure is therefore modified when they are present.

²⁸² Harold D. Appleton, Bert N. LaDu, Jr., Betty B. Levy, J. Murray Steele and Bernard B. Brodie, *J. Biol. Chem.* **205**, 803-13 (1953).

²⁸³ G. Smits, *Biochim. et Biophys. Acta* **26**, 424-7 (1957).

²⁸⁴ Makoto Hayashi, Tsutomu Unemoto and Komei Miyaki, *J. Pharm. Soc. Japan* **81**, 1039-41 (1961); *Chem. Pharm. Bull., Japan*, **10**, 535-5 (1962); Makoto Hayashi, Yoshinori Nakajima, Keizo Inoue and Komei Miyaki, *Chem. Pharm. Bull., Japan* **11**, 1200-2 (1963).

²⁸⁵ Donn J. Kushner, *Biochim. et Biophys. Acta* **20**, 554-5 (1956).

²⁸⁶ L. W. Wheeldon and F. D. Collins, *Biochem. J.* **70**, 43-5 (1958).

Beer's law is followed. Choline is also determined as the reineckate²⁸⁷ (Vol. IV, pp. 69-70). Micro concentrations of choline are precipitated from alkaline solution as choline reineckate, which is read in the ultra-violet in ammonium hydroxide solution.²⁸⁸ Acetone is replaced as a solvent by the ammonium hydroxide, because acetone absorbs in the ultraviolet. Beer's law is followed for 0.001-0.016 mg. per ml. 2-Dimethylaminoethanol does not interfere. An alternative is to develop the reineckate with ferric nitrate in nitric acid.²⁸⁹

For determination of free and bound choline in feeds, hydrolysis and extraction are first carried out with barium hydroxide-methanol-chloroform. The choline is then isolated from the hydrolysate by adsorption on a Florasil column. On the column, it is converted to a pink band of the reineckate, and excess reagent is washed out. The reineckate is eluted with acetone and read at 526 m μ .²⁹⁰ Chlorophyll interferes unless removed in a separate step. Choline as the reineckate in acetone is unreliable with fish lipids.²⁹¹

A method of determination of quaternary ammonium compounds with *cis*-aconitic anhydride is applicable to free and bound choline.²⁹² The latter need not be hydrolyzed to free choline. A longer time and higher temperature is required to convert the initial red-purple to green. Thereafter, it is read at 530 m μ . Phosphorylcholine is in Chapter 8.

Sample—Fat. Hydrolyze a 2-42-mg. sample, containing 2-12 micro-moles of choline by heating at 100° with 2 ml. of 1:1 hydrochloric acid and 2 ml. of 95% ethanol, for 18 hours. During the first hour of hydrolysis, use an air condenser, and as the ethanol is gradually lost, replace it with

²⁸⁷ H. J. Castro-Mendoza and J. del Rio Sanchez, *Rev. clin. espan.* **28**, 44-5 (1948); *Excerpta Med., Sect. II* **2**, 50 (1949); V. E. Munsey, *J. Assoc. Offic. Agr. Chemists* **35**, 766-9 (1953); Jean Louis Delsal, *Compt. rend.* **244**, 2855-8 (1957); K. Basu and B. N. Dutta, *J. Inst. Chem., India* **34**, 142-59 (1962); cf. Celia Levine and Edwin Chargaff, *J. Biol. Chem.* **192**, 465-79 (1951).

²⁸⁸ C. J. Ackerman, *Anal. Biochem.* **1**, 337-43 (1960).

²⁸⁹ Amalia Chervonagura, *Publ. Inst. Invest. Microquim., Univ. Nac. Litoral* (Rosario, Arg.) **24**, 125-36 (1958).

²⁹⁰ F. Lin and E. D. Schall, *J. Assoc. Official Agr. Chemists* **47**, 501-3 (1964); cf. C. J. Ackerman and W. D. Salomon, *Anal. Biochem.* **1**, 327-36 (1960); C. J. Ackerman and May Chou, *ibid.* **1**, 337-43 (1960).

²⁹¹ David Glick, *J. Biol. Chem.*, **156**, 643-51 (1944); C. Enternman, Alvin Taurog and I. L. Chaikoff, *J. Biol. Chem.* **155**, 13-18 (1944).

²⁹² C. J. F. Boettcher, C. Pries and C. M. van Gent, *Rec. Trav. Chim.* **80**, 1169-78 (1961).

water. Evaporate the hydrolysate to dryness *in vacuo* to remove excess hydrochloric acid. Add 2 ml. of water and adjust the pH to 6-7 with 4% sodium hydroxide solution. Remove fatty acids by extraction with diethyl ether. Filter the aqueous layer and wash the filter with 5 ml. of water. Evaporate the filtrate to dryness and dissolve the residue in 2 ml. of water. Develop choline as choline phosphomolybdate.

Dinitrophenylated lipids. Hydrolyze a 5-25 mg. sample by refluxing with 5 ml. of 1:10 hydrochloric acid in 95% ethanol for 3 hours. Evaporate the dryness *in vacuo* to remove excess hydrochloric acid. Add 1 ml. of water and extract 3 times with 5-ml. portions of 40-60° boiling petroleum ether. Reserve this extract. Extract the aqueous layer with three 5-ml. portions of diethyl ether and discard the ether extracts. Combine the petroleum ether extract with the aqueous layer. Add 1 ml. of 5:1 hydrochloric acid to bring the normality to approximately 6. Hydrolyze for 10 hours at 100°. Follow the procedure for fat, starting at "During the first hour of hydrolysis . . ." except that, in this case, it is petroleum ether that is being lost.

*Flour, semolina, noodles, or macaroni.*²⁹³ Grind a sample containing 2-10 mg. of choline to 20 mesh or finer. Add 2 grams of Filtercel, mix, and transfer to a Soxhlet extraction thimble measuring approximately 30 × 77 mm. Cover with a cotton pad, place in Soxhlet extractor, and add 150 ml. of methanol. Extract for 8 hours. Filter through a 20-mm. × 11-cm. extraction tube having a 10-cm. stem containing an asbestos pad covered with a 10-mm. layer of sand. Filter with suction. Rinse the flask and tube with methanol.

Evaporate to dryness at 100° with the aid of an air blast. Rinse down the sides with 2 ml. of methanol and add 20 ml. of 5:3 hydrochloric acid. Reflux for 1 hour. Wash the condenser with 5 ml. of hot water. Cool, and add 2 ml. of decolorizing carbon. Mix well, add 4 grams of Filtercel and mix. Prepare a Büchner-type medium porosity fritted glass funnel of 60-ml. capacity by adding with suction a suspension of 4 grams of Filtercel and 25 ml. of water to form uniform layer. Filter with suction. Rinse the digestion flask through the funnel and dilute to 40 ml. with water. Neutralize with 50% potassium hydroxide, using litmus paper. This requires approximately 12 ml. of alkali. Add 20 ml. of a solution prepared by dissolving 18 grams of barium hydroxide in 400 ml. of warm water, cooling, and diluting to 500 ml.

²⁹³ V. E. Munsey, *J. Assoc. Offic. Agr. Chemists* **36**, 766-9 (1953).

Add 1-2 drops of 1% alcoholic thymolphthalein solution. Acidify with glacial acetic acid until the blue just disappears, and bring back a faint tinge of blue with the barium hydroxide solution. Cool in a refrigerator for 1 hour or more. Add 2 grams of Filtercel, mix and filter on the same type of Büchner funnel as before. Wash with water up to 100 ml. Determine as the reineckate.

Tissues. Homogenize a sample containing 0.001-0.016 mg. of choline for 1 minute in 0.15 *M* tris(hydroxymethyl)aminoethane-hydrochloride buffer at pH 7.4. Centrifuge at 3000 rpm. for 5 minutes. Dilute the supernatant liquid to 3 ml. Add 0.53 ml. of concentrated nitric acid. Cover, and heat on a hot plate for 2.5-4.5 hours in subdued light to hydrolyze. Cool to room temperature. Neutralize slowly to the pink end point of phenolphthalein, using sodium hydroxide pellets followed by 40% sodium hydroxide solution. Add a calculated amount of 8% sodium hydroxide solution to bring the concentration to 0.1-0.2 *N* with respect to sodium hydroxide. Cool to room temperature. Filter with suction, and wash the beaker and wall of the funnel with water until the total volume of filtrate and washings reaches 5 ml. Determine as the reineckate.

Phospholipid. For hydrolysis, heat 50-200 mg. of phospholipid in 2-12 ml. of 1:5 hydrochloric acid in a sealed ampoule at 120° for 24 hours. Open and filter. Thoroughly extract the residual tar with hot water. If tar passes through the filter, extract it from the aqueous layer with hexane. If necessary to destroy amino acids, add 0.02 ml. of 2% sodium nitrite solution and a drop of 1:2 hydrochloric acid. Heat at 100° for 15 minutes and cool. Evaporate at 100° and dry in a vacuum desiccator. Take up in a known amount of water, 1-4 ml., and determine as molybdenum blue by the micromethod.

Procedure—As *choline periodide. Macro.* To prepare potassium triiodide, dissolve 15.7 grams of iodine and 20 grams of potassium iodide in water and dilute to 100 ml. A suitable sample consists of 2 ml. of an aqueous solution containing 0.005-0.75 mg. of choline contained in a pointed centrifuge tube with a ground stopper. Chill to 0-4°. Add 1 drop of 5% solution of an anion-active surfactant to eliminate small crystals of choline periodide that would accumulate on the surface of the liquid after centrifuging. Add 0.4 ml. of potassium triiodide reagent at 0-4°. Mix, and let stand for 30 minutes at 0-4°. Centrifuge for 25 minutes at 0-4°. Aspirate the supernatant liquid to remove excess reagent. Dissolve the

precipitate of choline periodide with 4 ml. of 0.5% iodine solution in ethylene dichloride. Add 1 ml. of water and shake vigorously for at least 40 seconds to reduce the effect of any remaining reagent. Let stand for 90 minutes at room temperature, shaking vigorously every 30 minutes. Centrifuge for approximately 10 minutes or until the ethylene dichloride layer is clear. Read a portion of the ethylene dichloride layer at 365 $m\mu$ against 4 ml. of 0.5% iodine solution in ethylene dichloride, which has been shaken with 1 ml. of water.

Micro. To 1 ml. of sample containing 0.12-1.8 mg. of choline, add 0.2 ml. of 0.5 *M* acetate buffer for pH 5. If betaines are present, repress them by replacing this buffer with a 0.5 *M* disodium phosphate one. Add 0.1 ml. of 1.66% potassium iodide solution. Shake with 4 ml. of 0.5% solution of iodine in ethylene dichloride. Centrifuge, and read the solvent layer at 385 $m\mu$ against a reagent blank.

As choline phosphomolybdate. Macro. To a sample containing 0.3-3 micromoles of choline, add 0.1 ml. of a saturated phosphomolybdic acid solution, which has been clarified by centrifuging. Let stand for 30 minutes at 5°. Centrifuge, and decant the supernatant liquid. Invert the tube and drain for 15 minutes. Suspend the precipitate in 1 ml. of isobutanol and centrifuge. Discard the supernatant liquid and repeat with another 1-ml. portion of isobutanol. Dissolve the precipitate in 3 ml. of acetone. Add 1 ml. of 1:3 sulfuric acid and 0.05 ml. of 5% stannous chloride solution in 5:1 hydrochloric acid. Dilute to 10 ml. with 95% ethanol and read the blue color at 630 $m\mu$.

*Micro.*²⁹⁴ Take a sample containing 0.1-4 micromoles of choline. If over 0.2 ml., concentrate to that level. Add 0.1 ml. of 10% phosphomolybdic acid. A yellow precipitate should appear immediately. Chill in ice for 1 hour to minimize the loss by solubility of the precipitate. Centrifuge. Pipet off the upper layer. Wash with 1 ml. and 1 ml. of 10% sodium sulfate solution at under 10°, without stirring up the precipitate, but centrifuging in each case.

As the reducing agent, dissolve 0.5 gram of 1-amino-2-naphthol-4-sulfonic acid by stirring in 200 ml. of freshly prepared 15% solution of anhydrous sodium bisulfite. Add 1 gram of anhydrous sodium sulfite. Filter, store in a dark bottle, and prepare weekly.²⁹⁵

²⁹⁴ A. J. De Koning, *Anal. Chim. Acta* **29**, 510-16 (1963).

²⁹⁵ Grant R. Bartlett, *J. Biol. Chem.* **234**, 466-8 (1959).

After removal of the wash liquids, dilute to 4.1 ml. with water and add 0.5 ml. of 1:25 sulfuric acid. Dissolve with stirring at 100°. Cool, and add 0.2 ml. of aqueous 5% ammonium molybdate tetrahydrate and 0.2 ml. of the reducing solution. Heat at 100° for 7 minutes and cool. Dilute to 50 ml. with water and read at 830 $m\mu$ against a reagent blank.

As the reineckate. Direct reading. The purified sample has been diluted to 100 ml. with water. Add 6 ml. of 2% ammonium reineckate solution in methanol and let stand overnight in a refrigerator. Collect the pink choline reineckate by suction in a Knorr extraction-type tube, 20 mm. \times 11 cm., having a 10-cm. stem on an asbestos mat, 3-4 mm. thick, covered with a 10-mm. layer of sand. Wash with 4-5 ml. portions of ice-cold water and with three 2.5-ml. portions of *n*-propanol. Discontinue suction when the sand is dry. Dissolve the precipitate with 5-ml. portions of acetone and drain without suction. Dilute to 25 ml. with acetone and read at 526 $m\mu$.

Tissue. The hydrolyzed sample has been diluted to 5 ml. with water. Add 2 ml. of 5% ammonium reineckate solution and chill for 4 hours. Centrifuge the reineckate precipitate at 3000 rpm. for 10 minutes. Wash the precipitate 3 times by suspension in 1 ml. of cold 1-propanol. Centrifuge and dry over sulfuric acid in the dark. Dissolve the precipitate in 1 ml. of acetone and centrifuge. Evaporate a 0.1-ml. portion of the acetone. Add 5 ml. of concentrated ammonium hydroxide. Dilute to 10 ml. with water, shake, and read at 303 $m\mu$. Calculate choline as follows:

$$\text{Micrograms of choline chloride in the sample} = \frac{A_{303}}{0.041} \times \frac{139.6}{422.7}$$

$$\times \text{vol.} \times 10 = 8.09 \times A_{303} \times \text{vol.} \times 10$$

in which A_{303} is the observed absorbancy at 303 $m\mu$; 0.041 is the specific absorbancy index for choline reineckate, where concentration is expressed in $\mu\text{g. ml.}$; 139.6 and 422.7 are molecular weights of choline chloride and choline reineckate, respectively; and vol. is the volume in ml. of ammonium hydroxide used to dilute the 0.1 ml. aliquot.

Feed. Total choline. As an extractant saturate 100 ml. of methanol by shaking 5-10 minutes with 4-5 grams of anhydrous barium hydroxide. Add 10 ml. of chloroform and decant from excess barium hydroxide.

Transfer a sample containing 5-50 mg. of choline to a Goldfish extraction thimble. If considerable chlorophyll is present, as in alfalfa

samples, percolate 50-100 ml. of methanol-free methyl acetate through the thimble at room temperature. If the chlorophyll is small, it is removed at a later step. Add 30 ml. of extractant and reflux at 2-4 drops per second for 4 hours at the high setting on the extractor. Cool and filter, using several portions of 1:10 glacial acetic acid-methanol for the transfer and to wash the filter. Determine that the filtrate is pH 2-6, adding more acetic acid if necessary. Dilute to 100 ml. with methanol.

Use a chromatographic column about 25 cm. \times 7.5 mm. with a 9 \times 2.5-cm. reservoir at the top. This reservoir has a capacity of about 30 ml. Form a thin slurry in methanol of about 4 grams of Florasil activated at 650°. Swirl, and let stand 10-15 seconds. Pour off the suspended dust and fine particles. Use additional methanol to transfer to the column over a glass wool plug, forming a depth of about 18 cm. Maintain methanol in the column until it is used.

Transfer an aliquot of the hydrolysate containing 2-5 mg. of choline to the column. As the last of it passes into the column, rinse down the reservoir with 5 ml. of methanol.

As a reagent, prepared fresh daily, shake 2-3 grams of ammonium reineckate with 100 ml. of water for 10 minutes. Filter out excess salt. Add 10 ml. of methanol to the column. If a green band due to chlorophyll or its degradation products appears, elute it with 20-25 ml. of methanol-free methyl acetate, using more if necessary. Add successively 10 ml. of 10% acetone, 5 ml. of ammonium reineckate reagent and 10 ml. of glacial acetic acid. Each reagent must just enter the bed before the next is added. Elute the pink band of choline reineckate with 10 ml. of acetone. Dilute the eluate to 10 ml. and read at 526 $m\mu$ against acetone.

Free choline. Follow the above procedure, but omit barium hydroxide from the extraction medium and reduce the time for extraction to 1 hour.

*By ferric nitrate.*²⁹⁶ *Food.* Extract a solid sample with 20 ml. of absolute ethanol, or a liquid sample with 20 ml. of 3:1 ethanol-ether. Saponify an appropriate aliquot with 1 ml. of 8% sodium hydroxide for 3 hours. Acidify with 1 ml. of 1:5 hydrochloric acid and evaporate to dryness. Extract with six successive 0.5 ml. portions of 1:16 hydrochloric acid. Filter the combined extracts and add 3 ml. of saturated ammonium reineckate solution. Chill in ice, centrifuge, and decant. Wash out excess ammonium reineckate with ice-cold ethanol.

²⁹⁶ Eduardo M. Barilari, Juan Abuin and Mirta Piedrabuana, *Publ. Inst. Invest. Microquim., Univ. Nacl. Litoral (Rosario, Arg.)* 25, No. 23-24, 5-9 (1959-60).

Take up the residual salt in 20 ml. of water and add 2 ml. of 20% sodium hydroxide solution. Evaporate to 2 ml. The choline is now represented by chromium hydroxide and sodium thiocyanate. Filter, and wash the filter with 10 ml. of water. Neutralize the filtrate by dropwise addition of concentrated nitric acid. Dilute to a known volume. Add 5 ml. to 5 ml. of 1% ferric nitrate solution. Read at 474 m μ . Potassium thiocyanate may be used as the standard. One part corresponds to 1.9 parts of choline.

2-CHLOROCHOLINE

Follow the micromethod for choline by phosphomolybdate until the precipitate has been washed and the wash liquids removed.

Dissolve the 2-chlorocholine phosphomolybdate in 4.1 ml. of 0.4% sodium hydroxide solution, heating as may be necessary. Acidify with 1:25 sulfuric acid and add 0.5 ml. excess. Complete from "Dissolve with stirring at 100°."

ANTENYL, OXYPHENONIUM BROMIDE, DIETHYL (2-HYDROXYETHYL) METHYLAMMONIUM α -PHENYLCYCLOHEXANEGLYCOLLATE BROMIDE

The familiar bromophenol blue reaction is applicable to this quaternary compound.²⁹⁷

Procedure—To the sample containing about 0.5 mg. of oxyphenonium bromide, add a 0.1% solution of bromophenol blue at pH 5. Extract the resulting yellow complex with chloroform. Wash the chloroform layer with a 0.4% solution of sodium hydroxide. Dilute this aqueous phase with ethanol and read at 600 m μ .

CARBOXYTOLBUTAMIDE

After extraction with amyl acetate from an acid medium, carboxytolbutamide is determined with dinitrofluorobenzene.²⁹⁸

Sample—*Urine*. Dilute a 1-ml. sample to contain 2-10 mg. of carboxytolbutamide. Add 1 ml. of 1:1 hydrochloric acid and 6 ml. of amyl

²⁹⁷ W. Grabowicz, *Chem. Anal.* (Warsaw) **8**, 79-85 (1963).

²⁹⁸ J. M. Nelson, Inge O'Reilly and Thomas Chulski, *Clin. Chim. Acta* **5**, 771-6 (1960).

acetate. Rotate gently 50-75 times and centrifuge. To 4 ml. of the organic layer, add 1 ml. of 0.1% dinitrofluorobenzene solution in amyl acetate and heat at 150° for 5 minutes. Cool and read at 380 m μ .

SPHINGOSINE, 2-AMINO-4-OCTADECANE-1,3-DIOL

Oxidation of the sphingosine base produces a higher fatty aldehyde which is determined with lead tetraacetate.²⁹⁹ On filter paper, sphingosine forms an orange red spot when sprayed with 0.25% fluorescein-sodium solution.³⁰⁰ The reaction must occur in neutral solution; acidity causes dissolving of the color from the paper; alkalinity prevents color formation. Up to 1.5 microgram of sphingosine and *dihydrosphingosine* can be measured and Beer's law is followed for 0.004-0.16 mg. sphingosine. *N*-Acetylsphingosine, *N*-acetyldihydrosphingosine, triacetylsphingosine, and triacetyldihydrosphingosine do not react. Agmatine, aniline, choline, diphenylamine, ethanolamine, and lysine do not form color with the reagent. The colors formed with adrenaline, arginine, betaine, histidine, methylamine, and trimethylamine can be removed from the paper by washing with water.

Sphingosine forms a complex with methyl orange, sodium *p*-dimethylaminoazobenzene sulfonate. Choline, galactosamine, and neuraminic acid, the hydrolysis or methanolysis products of lipids, do not interfere.³⁰¹ The sensitivity of the method is increased by extracting the ethyl acetate solution of sphingosine with sulfuric acid.

Sample—Tissues. To prepare Schiff's reagent, dissolve 1 gram of fuchsin in 700 ml. of boiling water, cool to room temperature, and filter. Add 50 ml. of 1:5 hydrochloric acid and 5 grams of sodium bisulfite, mix thoroughly and dilute to 1 liter with water. Let cool and stand in the dark until the solution is completely decolorized. To prepare sulfur dioxide solution, follow the procedure for Schiff's reagent, omitting the fuchsin.

Grind a fresh sample with ice. Add 50 ml. of ethanol per gram of sample. Reflux for 1 hour and filter. Treat the filtered residue with the same amount of ethanol as before, reflux, and filter. Evaporate the combined ethanol extracts to dryness under reduced pressure, using a stream of nitrogen. Dissolve the residue in chloroform. Evaporate a chloroform aliquot containing 0.3-2 mg. of sphingolipids to dryness at 100° under

²⁹⁹ Toshio Sakagami, *J. Biochem.* **45**, 313-7 (1958).

³⁰⁰ Kunihiro Saito, *J. Biochem.* **47**, 573-80 (1960).

³⁰¹ Carl J. Lauter and Eberhard G. Traus, *J. Lipid Research* **3**, 136-8 (1962).

reduced pressure. Reflux with 10 ml. of saturated barium hydroxide solution at 100° for 6 hours to hydrolyze the sphingolipids. Add 1 ml. of concentrated hydrochloric acid and continue the hydrolysis for 6 hours. Cool to room temperature, make the solution alkaline with 4% sodium hydroxide solution, and extract the sphingosine base three times with a total volume of 30 ml. of chloroform. Evaporate the combined chloroform extracts to dryness under reduced pressure. Dissolve the residue in benzene. Develop with lead tetraacetate.

Lipids. Evaporate a sample containing 0.01-0.1 micromole of sphingosine to dryness under a stream of nitrogen. Dissolve in 1:1100 hydrochloric acid and make the solution alkaline with 4% sodium hydroxide solution. Dilute to 2 ml. with water. If the sample is obtained by methanolysis, the concentration of methanol should not exceed 20% of the final volume. Extract sphingosine with 5 ml. of ethyl acetate, centrifuge, and wash with water. Develop with methyl orange.

Procedure—By lead tetraacetate. To a benzene aliquot containing 0.03-0.5 mg. of sphingosine base, add 10 mg. of lead tetraacetate crystals and oxidize for 10 minutes at 75°. Add a few drops of glycerol and continue heating to completely reduce excess lead tetraacetate. Cool to room temperature. Add 20 ml. of petroleum ether and 1 ml. of water. Mix. Separate the ether layer and reextract the aqueous layer with 5 ml. of petroleum ether. Evaporate the combined ether and petroleum ether extracts to dryness under reduced pressure. Dissolve the residue in 1 ml. of glacial acetic acid and add 10 ml. of Schiff's reagent. Stopper with a glass stopper and let stand for 24 hours. Add 10 ml. of sulfur dioxide solution and mix well. Extract by shaking vigorously with 10 ml. of isoamyl alcohol. After 2 minutes, centrifuge for 10 minutes. Read the isoamyl alcohol layer at 550 m μ and subtract a blank.

By methyl orange. Evaporate the sample to dryness under nitrogen and dissolve the residue in 1 ml. of 1:1100 hydrochloric acid. Make the solution alkaline with 4% sodium hydroxide solution and dilute to 2 ml. with water. In extracts containing sphingosine obtained by methanolysis, the content of methanol should not exceed 20% of the final volume.

Extract the solution with 5 ml. of ethyl acetate and wash the extract with water. Treat the ethyl acetate solution with 2 ml. of 0.01 *M* acetate buffer, at pH 3.65, which has been previously equilibrated with ethyl acetate. Add 0.1 ml. of 0.5% methyl orange solution. Shake for 1 minute.

Centrifuge, and read the organic phase at 415 $m\mu$. Alternatively, treat 2-4 ml. of the organic phase with 0.5-2 ml. of 1:35 sulfuric acid and read the acid phase at 515 $m\mu$.

PENICILLAMINE

Penicillamine forms a blue color with ferric chloride, which is stabilized with potassium cyanide.³⁰² Krebs-Ringer bicarbonate buffer, sucrose, oxidized penicillamine, or reduced glutathione do not interfere. L(+)-Cysteine gives 5-7% of the optical density produced by penicillamine. Beer's law is followed.

Procedure—Dilute a solution containing 0.1-0.8 micromoles of penicillamine hydrochloride to 2 ml. with water. Add 0.5 ml. of 1.3% potassium cyanide solution and 0.5 ml. of 2.4% ferric chloride solution. Mix, and heat at 65° for 5 minutes. Cool and dilute to 5 ml. with water. Centrifuge, and read the blue supernatant liquid at 645 $m\mu$ against a reagent blank.

CAPSAICINE, 8-METHYL-N-VANILLYL-6-NONINAMIDE

Capsaicine in methanol is developed with 2,6-dichloro-*p*-benzoquinone-4-chlorimine³⁰³ (cf. Vol. IV, pp. 78-80).

Procedure—*Capsicum*. Extract the sample exhaustively with absolute methanol in a Soxhlet apparatus. Mix 5 ml. of extract with 40 ml. of a borate buffer for pH 9.4. Adjust to 20° and add 1 ml. of a 1% solution of 2,6-dichloro-*p*-benzoquinone-4-chlorimine. Dilute to 50 ml. with the buffer solution. After exactly 25 minutes in the dark, read at 595 $m\mu$ against water and correct for a blank determination.

CB1414, 4-bis(2-CHLOROETHYL)AMINO-2-METHYLAZOBENZENE-2'-CARBOXYLIC ACID

CB1414 absorbs in the visible region with a maximum at 525 $m\mu$.³⁰⁴

³⁰² Prabhat R. Pal, *J. Biol. Chem.* **234**, 618-19 (1959).

³⁰³ Pharmaceutical Society and Society for Analytical Chemistry. Joint Committee on Methods of Assay of Crude Drugs, *Analyst* **89**, 377-88 (1964).

³⁰⁴ J. H. Linford, *Biochem. Pharmacol.* **11**, 973-4 (1962).

Procedure—*Serum*. Shake 1 ml. of serum with 20 mg. of sodium bicarbonate. Add 10 ml. of absolute ethanol to precipitate proteins. Centrifuge. Evaporate the solvent under reduced pressure. Dissolve the residue in 5 ml. of 0.1 *M* phosphate buffer for pH 3.4-3.6. Extract with 5, 5, and 5 ml. of chloroform. Evaporate the combined extracts *in vacuo*. Dissolve the residue in 0.3 ml. of 1:1 hydrochloric acid, more if necessary. Add 9 times this volume of absolute ethanol. If necessary, dilute further with 1:1 hydrochloric acid and ethanol and read at 525 m μ .

SPERMIDINE, *N*-(3-AMINOPROPYL)-1,4-BUTANEDIAMINE

This polyamide can be enzymatically converted to propane-1,3-diamine Δ' -pyrroline by spermadine-adapted cells. The latter is then determined by *o*-aminobenzaldehyde, forming 2,3-trimethylene-1,2-dihydroquinazolium.³⁰⁵ There is no significant interference by spermine, 3,3'-diaminodipropylamine, propane-1,3-diamine, butane 1,4-diamine, and corresponding diamines to decane-1,10-diamine.

Sample—*Rat liver*. Suspend 6.7 grams in 130 ml. of 6.5% trichloroacetic acid solution. Homogenize for 5 minutes. Filter after 5 minutes at 100°. Extract with four successive 200-ml. portions of ethyl ether to remove the trichloroacetic acid. Evaporate the aqueous phase to dryness at 100° and take up in 20 ml. of water.

Procedure—Prepare a spermidine-yeast extract medium containing per liter 0.5 gram of Difco yeast extract, 2 grams of dipotassium phosphate trihydrate, 1 gram of monopotassium phosphate, 1 gram of glucose, 0.2 gram of magnesium sulfate heptahydrate, and 0.1 gram of spermidine trihydrochloride. Grow *Serratia marcescens*, either J or ATCC-8195, in 2-liter flasks, containing 1 liter of medium, at 30° for about 20 hours. Harvest by centrifuging at 4°. Wash three times with 0.9% salt solution and freeze-dry over phosphorus pentoxide. Store at -20° or under vacuum at room temperature.

To 0.1-0.3 ml. of sample add 0.5 ml. of 0.05 *M* phosphate buffer for pH 6.6. Suspend 5 mg. of cells in 1 ml. of 0.9% sodium chloride solution. Add 0.1 ml. of this to the sample and dilute to 0.9 ml. with the salt solution. Incubate at 37° for 30 minutes and add 0.1 ml. of 0.1% *o*-aminobenzaldehyde solution. Incubate at 37° for 30 minutes and read at 435 m μ .

³⁰⁵ Uriel Bachrach and I. S. Osier, *J. Biol. Chem.* 238, 2098-2101 (1963).

ETHINAMATE, ETHYNYLCYCLOHEXYL CARBAMATE

Ethinamate is oxidized by bromine and coupled with diazotized sulfanilic acid.³⁰⁶ Vanillin-sulfuric acid reagent is also used for determination of this test substance.³⁰⁷ It follows Beer's law for 0.1-0.5 mg. per ml. Amobarbital, allobarbital, pentobarbital, tinopental, and phenobarbital show similar color reactions with the latter reagent, but can be separated by reading a narrow band. For extraction of the test substance from urine, use dichlorethylene made alkaline with ammonia.

Procedure—*By oxidation.* To 5 ml. of an aqueous solution of ethinamate containing 0.005-0.1 mg., add 0.5 ml. of 0.32% bromine solution and shake for 10 minutes. Add 0.2 ml. of 0.1% sodium nitrite solution to destroy the excess bromine. Add 1.5 ml. of 0.1% diazobenzenesulfonic acid and 2.5 ml. of 0.8% sodium hydroxide solution. Mix, and heat at 40° for 30 minutes. Add 1 ml. of pyridine and shake. Read at 400 m μ .

By vanillin-sulfuric acid. To 1 ml. of methanolic sample add 2 ml. of 2% vanillin solution in 1:1 sulfuric acid. Heat at 100° for 20 minutes. Cool and dilute to 10 ml. with water. Read at 530 m μ .

SEGONTIN, *N*-(1-METHYL-2-PHENYLETHYL)-3,3-DIPHENYLPROPYLAMINE

After extraction from acid solution by chloroform, segontin is read in the ultraviolet.³⁰⁸ It is also read as the reineckate in acetone and by Tropaeolin 00, diphenylamine orange.

Procedure—This particular technic is not applicable to biological fluids. To a 5-ml. sample containing 0.1-2 mg. of segontin, add 1 ml. of 1:5 hydrochloric acid. Extract with 5 ml. of chloroform and read the organic layer at 259 m μ .

Alternatively, mix 5 ml. of sample containing 0.3-1.8 mg. of segontin with 3 ml. of cold-saturated ammonium reineckate solution. Filter, and wash with very dilute reineckate solution. Dry at 90° for 1 hour. Dissolve in acetone and read at 545 m μ .

Urine. Mix 5 ml. of sample containing 0.2-2 mg. of segontin with 5 ml. of acetate buffer for pH 4.62 and 3 ml. of 1% aqueous Tropaeolin 00.

³⁰⁶ Masaru Nakano, *Arch. Pract. Pharm., Japan* **22**, 158-60 (1962).

³⁰⁷ Hajime Numatri and Ikuo Endo, *Shinshu Igaku Zasshi* **10**, 258-63 (1961).

³⁰⁸ A. Haussler, *Arzneimittel-Forsch.* **10**, 585-8 (1960).

Extract with four 5-ml. portions of chloroform. Acidify the combined chloroform layers with 1% sulfuric acid in methanol and dilute to 50 ml. with chloroform. Read at 545 m μ .

Scrum. To precipitate albumin, mix a 5-ml. sample with 2 ml. of ethanol. Centrifuge, and treat 5 ml. as described for urine.

2'-(β -DIETHYLAMINOETHOXY)-3-PHENYLPROPIOPHENONE

This compound is precipitated from aqueous solution as the reineckate or the phosphomolybdate. These are then dissolved in acetone for reading.³⁰⁹

Procedure—Dissolve a sample containing 10-50 mg. of the test substance by heating with 20 ml. of water for 5 minutes at 100°. Acidify to Congo red by dropwise addition of 1:10 hydrochloric acid. Add, dropwise, 6 ml. of a saturated solution of ammonium reineckate. Filter after 1 hour. Wash the precipitate with 1:10 hydrochloric acid. Dissolve the precipitate in acetone, dilute to 25 ml., and read at 395 m μ .

If phosphomolybdic acid is substituted for ammonium reineckate, read the solution at 400 m μ or 450 m μ .

ETHOPABATE, METHYL-4-ACETAMIDO-2-ETHOXYBENZOATE

Ethopabate is first extracted from the feed with chloroform. Interfering substances such as primary aromatic amines, procaine, etc., are extracted with dilute hydrochloric acid. Thereafter, controlled acid hydrolysis converts ethopabate to a free amine. Fatty and colored substances are extracted by hexane for discarding. The free amine is then diazotized, and coupled with *N*-(1-naphthyl)-ethylenediamine. The color body is extracted with butanol for reading.³¹⁰

There is no interference by *p*-aminobenzoic acid, arsanilic acid, 2-acetylamino-5-nitrothiazole, amprolium, glycarbylamide, 3,5-dinitrobenzamide, furazolidone, nitrophenide, 3-nitro-4-hydroxyphenyl arsonic acid, nitrofurazone, nithazide, piperazine, procaine penicillin, streptomycin, or zoalene. There is interference by sulfaquinoxaline, nicarbazin, and acetyl-(*p*-nitrophenyl)-sulfanilamide of which the latter two form primary amines on acid hydrolysis.

³⁰⁹ J. Giusti and G. F. Di Paco, *Boll. Chim. Farm.* **100**, 425-9 (1961).

³¹⁰ C. R. Szalkowski, *J. Assoc. Off. Agr. Chem.* **47**, 221-5 (1964); cf. *ibid.* **46**, 100 (1963); *ibid* **46**, 464-7 (1963)

Procedure—*Feed*. Grind the sample to pass a No. 20 sieve. Transfer a sample to contain about 0.06 mg. of ethopabate and add 100 ml. of chloroform. Reflux for 2 hours, using a magnetic stirrer. Filter, avoiding loss of solvent by evaporation.

Mix 25 ml. of the extract with 15 ml. of 1:40 hydrochloric acid and shake for 2 minutes. Centrifuge, draw off the aqueous layer without disturbing the interface, and discard. Repeat the washing with 10 ml. of the diluted hydrochloric acid. Finally, wash with 10 ml. and 10 ml. of water and discard the washings. These steps must be completed in less than an hour to avoid loss of the test substances by acid hydrolysis.

Transfer the chloroform layer containing ethopabate and wash the container with 3 ml. and 3 ml. of methanol. Evaporate to dryness at 100°. Take up the residue with 15 ml. of hexane and wash the container with 5 ml. of methanol. Further rinse the container with 10, 10, and 5 ml. of 1:40 hydrochloric acid. Shake the hexane-acid mixture for 1 minute and centrifuge. Discard the hexane layer without disturbing the interface. Wash with 10, 10, and 10 ml. of hexane, and discard each washing.

Add glass beads to the washed acid solution and heat at 100° for 45 minutes with the level of the liquid below that of the bath. Cool the residual solution to 20-25° and add 1 ml. of fresh 0.2% sodium nitrite solution. After 2 minutes, add 1 ml. of 1% ammonium sulfamate solution. After 2 minutes, add 1 ml. of a 0.2% aqueous solution of *N*-(1-naphthyl)-ethylenediamine dihydrochloride. After 10 minutes, add 5 ml. of *n*-butanol and 5 grams of sodium chloride. Shake vigorously until the salt is dissolved, and centrifuge. Read the clear butanol layer at 555 m μ against butanol. Apply a correction for a reagent blank of 5 ml. of methanol and 25 ml. of 1:40 hydrochloric acid treated as for the sample, from "Add glass beads . . ."

DITHIOCARBAMATES, FERAM, FERRIC DIMETHYLDITHIOCARBAMATE; ZIRAM, ZINC DIMETHYLDITHIOCARBAMATE; MANEB, MANGANOUS ETHYLENE*bis*-(DITHIOCARBAMATE); ZINEB, ZINC ETHYLENE*bis*(DITHIOCARBAMATE); THIRAM, *bis*(DIMETHYLTHIOCARBAMOYL)DISULFIDE; METIRAM, A MIXTURE OF [ETHYLENE*bis*(DITHIOCARBAMATO)]ZINC AND [DITHIO*bis*(THIOCARBONYL)-IMINOETHYLENE]*bis*(DITHIOCARBAMATO)ZINC.

The determination of the above dithiocarbamates depends on acid decomposition to carbon disulfide and the corresponding amine. The carbon disulfide is absorbed in ethanolic cupric acetate and an alkylamine to give a yellow chelate.³¹¹

³¹¹ D. G. Clarke, Harry Baum, E. L. Stanley and N. F. Hester, *Anal. Chem.*, 23,

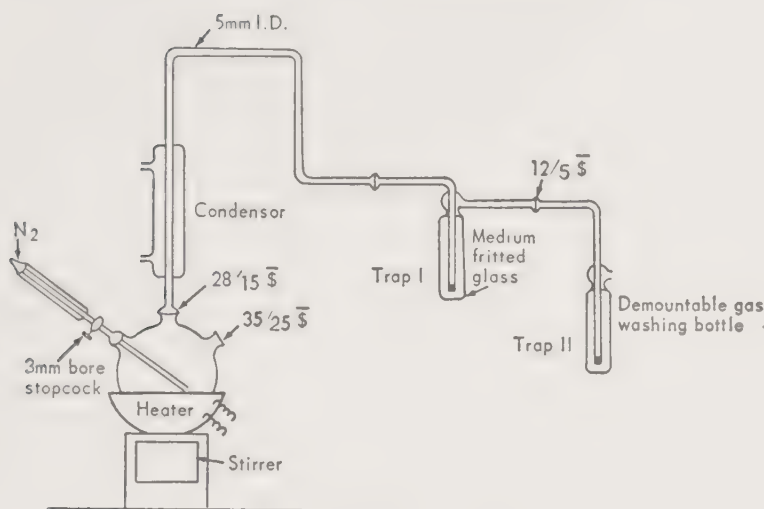


FIG. 5.

Decomposition-absorption apparatus for carbon disulfide

In the apparatus shown in Figure 5, the tip of the addition funnel extends to within 1 cm. of the bottom of the reaction flask to provide agitation of the sample by the incoming nitrogen during digestion. Only the zinc salt portion of the molecule in metiram releases carbon disulfide. The standard curve is prepared with carbon disulfide and related to the various dithiocarbamates by assay of the respective materials for carbon disulfide. The method will determine as little as 0.02 mg. of carbon disulfide accurately. Prompt action is essential when the dithiocarbamate makes contact with the sulfuric acid, and the sugars, etc., are released. Samples must either be analyzed promptly or stored in a frozen form. Only zineb gives over 0.5% of hydrogen sulfide as an alternate decomposition, and with it, only about 10% of the sulfur is evolved by that route.

The final solution has an equilibrium between a 1:1 and a 1:2 complex; therefore, the curve does not pass through the origin and an appropriate copper concentration must be present. Except for zineb, recoveries are 85-100%.

Procedure—Add 15 ml. of 20% zinc acetate solution to trap I to remove interfering gases. To trap II, add 12.5 ml. of an appropriate color

1842-6 (1951); W. K. Lowen, *ibid.* 23, 1846-50 (1951); H. L. Pease, *J. Assoc. Offic. Agr. Chemists* 40, 1113-18 (1957); R. F. Hauermann, *ibid.* 40, 264-70 (1957); Thomas E. Cullen, *Anal. Chem.* 36, 221-4 (1964).

reagent. For less than 0.2 mg. of carbon disulfide, add 25 grams of diethanolamine to 0.004 gram of cupric acetate monohydrate and dilute to 250 ml. with 95% ethanol. For 0.2-1 mg. of carbon disulfide, the reagent contains 0.012 gram of cupric acetate monohydrate.

Immediately add a sample of not over 200 grams, carrying a total dithiocarbamate residue equivalent to 0.05-1.0 mg. of carbon disulfide into the dry flask of the apparatus. At once add 150 ml. of boiling 1:2.5 sulfuric acid through the addition funnel. Pass nitrogen through the system at 6-10 ml. per minute to carry the evolved carbon disulfide. Heat the contents of the flask to boiling and continue the digestion for 45 minutes.

Dilute the contents of trap II to 25 ml. with ethanol and let stand for 15 minutes. Read at 435 $m\mu$ against the reagent, similarly diluted as a blank.

NYLON AMINES

The amine end groups of nylon are developed with 2,4-dinitrofluorobenzene for reading. The solvent is ethanolic lithium bromide and formic acid.³¹² The rate of reaction is speeded up by the presence of up to 15% of water. It is pH dependent. The reading at 440 $m\mu$ is a compromise between sensitivity and the reagent blank.

Procedure—Grind flakes or chips to pass 20-mesh. Yarn may be used as-is. Weigh up to 0.025 gram of sample containing no more than 2 microequivalents of amine. Dissolve 60 grams of lithium bromide in 300 ml. of absolute ethanol and 18 ml. of water. Reflux to dissolve, let stand overnight, and decant from any residue.

Reflux the sample with 3 ml. of the lithium bromide solution until dissolved. Add 0.3 ml. of 1:200 hydrochloric acid through the condenser. After refluxing for 1 minute to mix, remove from the heat. Remove the condenser and at once add 0.2 ml. of 10% solution of 2,4-dinitrofluorobenzene in absolute ethanol. Without letting it cool, add 0.03-0.05 gram of sodium bicarbonate. Swirl to mix and heat at 80° for 40 minutes. Before removing from the bath, add 1 ml. of 98-100% formic acid dropwise with mixing. Cool, dilute to 10 ml. with formic acid, and read at 440 $m\mu$ against a reagent blank.

³¹² R. G. Garmon and M. E. Gibson, *Anal. Chem.* **37**, 1309-12 (1965).

METHAPYRILENE, 2-[(2-DIETHYLAMINOETHYL)-2-THENYLAMINO]
PYRIDINE

Methapyrilene is determined by ninhydrin.³¹³ If dextrometorphan is present, it is preextracted from acid solution with chloroform. There is no interference by chlorpheniramine, mepyramine, phenylephrine, and tripeleennamine.

Procedure—Make a solution containing about 5 mg. of methapyrilene alkaline with 4% sodium hydroxide solution. Extract with 15, 10, 10, and 10 ml. of *n*-hexane. Filter the extracts and dilute to 50 ml. Evaporate 1 ml. of the extract by blowing it with nitrogen. Add 0.25 ml. of 1% ninhydrin solution in concentrated sulfuric acid. Rotate for 5 minutes so that all of the residue is treated. Add 5 ml. of 5% solution of Tween 80. Read at 525 $m\mu$ against a reagent blank.

LIDOCAINE, 2-DIETHYLAMINO-2',6'-ACETOXYLIDIDE

The copper complex of lidocaine in alkaline solution gives a stable purple color.³¹⁴ Antihistamines do not interfere. It is also read in chloroform at 6 μ .³¹⁵

Procedure—*Solutions.* To 5 ml. of sample, add 1 ml. of 1.3% solution of copper sulfate pentahydrate. Add 1 ml. of 15% sodium carbonate solution and centrifuge. Read at 570 $m\mu$.

Ointment. Shake a sample containing about 60 mg. of lidocaine with 20 ml. of chloroform and 10 ml. of 1:100 hydrochloric acid for 1 minute. Centrifuge, and extract the chloroform layer with 10, 10, and 10 ml. of 1:100 hydrochloric acid. Dilute to 50 ml. with 1:100 hydrochloric acid and develop as described for solutions.

³¹³ N. Scott, *J. Pharm. Sci.* **53**, 1121-2 (1964).

³¹⁴ Teishiro Kushima and Hiroshi Uno, *Archs. Pract. Pharm.* **24**, 251-3 (1964).

³¹⁵ H. M. Koehler and J. J. Hefferren, *J. Pharm. Sci.* **53**, 1126-7 (1964).

CHAPTER 3

AMINO ACIDS

CYSTEINE as the reduced form and cystine the disulfide are amino acids. Their multifunctional classification leads to their appearing primarily as sulfur derivatives in Volume III, page 473, and Volume IIIA, page 106. A method also appears in this chapter. Glutathione is another sulfur-containing acid. It appears in Volume III, pages 489-93, and Volume IIIA, pages 460-1. However, its determination by hydroxylamine hydrochloride and ferric chloride appears in this volume under γ -aminobutyric acid, page 273.

Methionine, as a sulfur-containing amino acid, appears in Volume III, pages 497-9, and Volume IIIA, p. 461. Acetylmethionine is separated from it by paper chromatography, using 4:1:5 butanol-acetic acid-water. After alkaline saponification, *acetylmethionine* is determined by sodium β -naphthoquinone sulfonate.¹ While the name of *pyroglutamic acid* suggests a close relation to glutamic acid, it is 2-pyrrolidone-5-carboxylic acid, a nitrogen-containing cycle, and appears in Chapter 6, page 455.

For *guanadineacetic acid*, *glycocyamine*, see arginine where it is simultaneously separated and determined by alkaline thymine- α -naphthol, on page 285. For a method for serine along with ethanolamine, see page 103. α,δ -*Diaminovaleric acid*, *2,5-diaminopentanoic acid*, *ornithine*, is determined by *o*-aminobenzaldehyde by a technic described under α -oxo- δ -aminovaleric acid, page 286. For the separation of *N*- α -*acetylkynurenine* from related tryptophan metabolites and its fluorescent reading, see *kynurenine*, page 295.

Ninhydrin partially reduced by dithionate will measure 0.1-10 microgram of *leucine* nitrogen per ml.² In the range of 1-3 micrograms, it is accurate to $\pm 2\%$.

Primary amines and the amino and imino groups of amino acids and peptides react with 2,4,6-trinitrobenzene-1-sulfonic acid to give a peak in the vicinity of 340 $m\mu$.³

The reaction with anthrone is applicable to determination of *S*-(car-

¹ J. Bednář, *Ceskoslov. farm.* **5**, 37 (1956).

² Hermann Stegemann, *Z. Physiol. Chem.* **319**, 102-9 (1960).

³ Kazuo Satake, Tsuneo Okuyama, Mochihiko Ohashi and Tomotaka Shimoda, *J. Biochem. (Tokyo)* **47**, 654-60 (1960).

benzylmethyl)cysteine.⁴ As applied to proteins, 0.02% of anthrone in 2:1 sulfuric acid at 100° for 1 hour gives an orange-red color with maxima at 443 and 542 m μ .

The reaction of nitric acid with 1,3-indanedione gives 2-nitroso-1,3-indanedione. Heated with aqueous or alcoholic amino acids, this reacts much like ninhydrin.⁵ A typical reaction is 5 mg. of reagent with 0.1 millimole of amino acid in 10 ml. of citrate buffer for pH 4.5 at 120° for 30-230 minutes. Reaction occurs with *glycine*, *histidine*, *tryptophan*, *serine*, and *phenylalanine*. None occurs in 5 hours with *alanine*, α -amino-butyric acid, *norvaline*, *norleucine*, *valine*, *leucine*, *isoleucine*, *glutamic acid*, and β -alanine. *Ornithine* gives a black precipitate.

7-Aminoheptanoic acid, ω -amino-oenanthic acid, forms a red compound with hydroquinone in methanol.⁶ This is read at 485 m μ .

Acid 8-hydroxyquinoline, used as a specific reagent for *monosubstituted guanidines* such as *arginine*, also gives a red color with *aspartic acid*.⁷ The color is less sensitive than for *arginine* and develops more slowly. Yellowish tints are given with this reagent by *asparagine*, *glutamic acid*, and *glutamine*.

Lanthionine is isolated from protein hydrolysates by descending paper chromatography, using fresh 150 grams of phenol:10 ml. of glacial acetic acid:40 ml. of water:40 ml. of ethanol.⁸ The patches are eluted with 0.1 M formate buffer for pH 3.42. Development is with 1.25 grams of ninhydrin in 2 parts of 6 M phosphoric acid and 3 parts of glacial acetic acid for reading at 450 m μ .

Tetrahydrofolic acid and *tetrahydro-N⁵,N¹⁰-methylenefolic acid* are determined by the reaction catalyzed by hydroxymethyltetrahydrofolate dehydrogenase.⁹ The change in molecular absorbance is read at 340 m μ . An alternative is to read the end product directly in 1:120 hydrochloric acid solution at 350 m μ .

Lysine forms a colored compound with ninhydrin at pH 1 but *norleucine*, ϵ -aminocaproic acid, does not. Both form colored compounds at pH 6.4. Therefore, *lysine* can be determined at pH 1, and by difference, *norleucine* at pH 6.4.¹⁰ For determination of *ornithine* as α -oxo-S-amino-valeric acid, see page 286.

⁴ M. A. Jermyn, *Anal. Biochem.* **5**, 433-42 (1963).

⁵ E. Neuzil and R. Masseyeff, *Bull. Soc. Pharm.* (Bordeaux) **100**, 55-61 (1961).

⁶ E. P. Usova and K. A. Snesev, *Zhur. Anal. Khim.* **19**, 243-7 (1964).

⁷ I. Carni-Catsadima, *Arch. Inst. Pasteur Hellenique* **7**, 123-4 (1961).

⁸ L. M. Dowling and W. G. Crewther, *Anal. Biochem.* **8**, 244-56 (1964).

⁹ B. V. Ramasastri and R. L. Blakley, *J. Biol. Chem.* **239**, 106-11 (1964).

¹⁰ K. Czerepko and N. Wolosowicz, *Talanta* **10**, 813-16 (1963).

α -AMINO ACIDS IN GENERAL

The reaction of decarboxylated and deaminated amino acids by ninhydrin remains the most common procedure for α -amino acid determination (cf. Vol. IV, p. 104). The purple product is soluble in all alcoholic solvents and solvents containing phenolic groups. It is insoluble in ether, chloroform, and nonpolar organic solvents. It completely decomposes in dilute aqueous solution and decomposes immediately in acidified solution. It is stable in alkaline solutions containing 0.1% ethylenediaminetetraacetic acid. It is also stable in ethanolic or *n*-butanol solutions containing ethylenediaminetetraacetic acid.

The sensitivity of the reaction is much greater in organic solvents than in water and is also influenced by the hydrogen-ion concentration in the solvent. Below pH 4 in aqueous solutions, no reaction occurs.¹¹

For continuous recording, the amino acid solution passes through a Dowex-50-X8 column at 50° and 30 ml. per hour. Ninhydrin solution is injected and it flows in 20 minutes through a 1-mm. \times 20-meter Teflon coil in boiling water. The absorption is then automatically recorded.¹² Stannous chloride has been added to the reagent to prevent oxidation. To avoid precipitation of tin salts, hydrindantin, the reduction product of ninhydrin, or ascorbic acid may be added in place of stannous chloride.¹³ Reduced ninhydrin reagents such as these must be stored under nitrogen. By preparation of the ninhydrin in cyanide solution, storage of the reagent under nitrogen is eliminated.¹⁴ This procedure gives greater sensitivity than with pyridine and more reproducible results. The color is stable for at least 1 hour. The results are low with tryptophan and high with lipene.¹⁵ Excess cyanide reacts with ninhydrin to produce an orange substance with measurable absorbance at 575 m μ .¹⁶ Addition of hydroquinone to the reagent in concentrations of 0.001-0.01% does not affect the color reaction and may prevent peroxide formation, which interferes.

Color is enhanced by addition of disodium ethylenediaminetetra-

¹¹ H. Meyer, *Biochemical J.* **67**, 333-40 (1957); cf. Thadee Staron, Claude Allard and Marie Madeleine Chambre, *Compt. Rend.* **253**, 2280-2 (1961).

¹² K. Hannig, *Clin. Chim. Acta* **4**, 51-7 (1959).

¹³ Stanford Moore and Wm. H. Stein, *J. Biol. Chem.* **211**, 907-13 (1954); Takeo Tsukamoto, Tetsuya Komori and Nadao Kinoshita, *Pharm. Bull.* **5**, 63-6 (1957).

¹⁴ E. C. Cocking and E. W. Yemm, *Biochemical J.* **58**, xii (1954).

¹⁵ E. W. Yemm and E. C. Cocking, *Analyst* **80**, 209-13 (1955); cf. Michizo Suyama and Shoji Konosu, *Bull. Japan Soc. Sci. Fisheries* **23**, 555-60 (1957-8).

¹⁶ Harold Kalant, *Anal. Chem.* **28**, 265-6 (1956).

acetic acid in place of pyridine.¹⁷ This substitution improves reproducibility by eliminating trace metal interferences and yields lower blanks than the pyridine reagent. A separate buffer is used to maintain the pH at 5.1. Proline, hydroxyproline, and cysteine do not form purple color with the reagent. Although urea, creatinine, and hippuric acid all react, the interference even from large amounts of creatinine and hippuric acid is negligible. Solvents for ninhydrin vary widely. The ninhydrin-cyanide reagent is dissolved in methyl cellosolve. The methyl cellosolve used should give no more than a very faint peroxide test with 10% potassium iodide solution. Alternatively, to produce a more stable reagent, the cyanide is dissolved in sodium acetate buffer.¹⁸ A more stable reagent is made by adding the cyanide solution to the buffer just before use.¹⁹ Unmixed the cyanide solution is stable for 2 weeks. Under these conditions, urea and ammonium ion interfere.

With ethanol as a solvent, strong oxidizing and reducing agents and trichloroacetic acid interfere. In butanol, the reaction product is red and the absorption maximum is 530 m μ . With ethanol as the solvent in the presence of ethylenediaminetetraacetate, urea does not interfere. Ammonia is removed by distillation.²⁰ With ethylene glycol as the solvent, color development is complete in 6 to 10 minutes.²¹ Ninhydrin will determine 0.002 mg. of amino acids in water.²²

Paper chromatograms are developed with ninhydrin in a 1:9 mixture of 5% ethanol and acetone. After 10 minutes at 20°, this is heated for 60 minutes in air at saturated humidity. Thereafter, the spot is extracted with 0.42% sodium bicarbonate in 48% ethanol and read at 578 m μ .²³ An advantage of 0.2% ninhydrin in 2-4% acetic acid in acetone is that there is rapid drying and no spreading of the spots.²⁴

Tungstic acid is the usual protein precipitant. Ethanol, trichloroacetic

¹⁷ H. M. Rubinstein and J. D. Pryce, *J. Clin. Pathol.* **12**, 80-4 (1959).

¹⁸ Hyman Rosen, *Arch. Biochem. Biophys.* **67**, 10-15 (1957).

¹⁹ D. R. Grant, *Arch. Biochem. Biophys.* **6**, 109-110 (1963).

²⁰ Avedis Khachadurian, W. Eugene Knox and Andrea M. Cullen, *J. Lab. and Clin. Med.* **56**, 321-332 (1960); Thadee Staron, Claude Allard and Marie Madeleine Chambre, *Compt. Rend.* **253**, 2280-2 (1961); cf. Yu. N. Kremer and A. L. Valtner, *Lab. Delo* **2**, No. 6, 3-6 (1956).

²¹ D. Siest, G. Siest and S. Besson, *Bull. Soc. Pharm. Nancy* **51**, 19-23 (1961).

²² A. D. Semenov, I. N. Vivleva, and V. G. Datsko, *Trudy Komiss. Anal. Khim. Akad. Nauk. SSSR* **13**, 62-5 (1963).

²³ Peter Bohley, *Naturwissenschaften* **49**, 326 (1962).

²⁴ J. deWael and R. Diaz Cadavieco, *Rec. trav. chim.* **73**, 333-46 (1954).

acid, and sulphosalicylic acid are not satisfactory protein precipitating agents for this method unless the amino acid is later isolated. Although picric acid is satisfactory as a precipitant, it requires additional time to remove the excess. A somewhat similar method uses *N*-ethylmaleimide, alcoholic cyanide, and a borate buffer.²⁵

Serum or other fluid is deproteinized with trichloroacetic acid, and the filtrate is chromatographed on an alumina column.²⁶ Glutamic, aspartic, and keto acids are sorbed. Other amino acids pass through. Glutamic acid is eluted with 1:35 acetic acid and developed with ninhydrin. Aspartic acid and keto acids are eluted with 1:15 ammonium hydroxide and developed with diphenylamine. The other amino acids are separated by paper chromatography and developed with ninhydrin.

Equipment is available for automatically continuously cleaning up the solution on resin, developing the color with ninhydrin, reading it, and recording it.²⁷

After oxidation by ninhydrin the nitrogen converted to ammonia is determinable by Nessler's reagent.²⁸ As exceptions, lysine, histidine, and glutathione give high results, while cystine and cysteine give irregular nonreproducible results. The amide groups of asparagine and glutamine do not interfere. Tryptophan and proline give low values. Di- or tripeptides and carnosine give very low results.

Prior to color development, amino acids can be separated by electrophoresis on paper²⁹ as an alternative to paper chromatography.³⁰ As applied in 0.2 *N* acetate buffer at pH 3.8, electrophoretic separation gives as bands aspartic acid, glutamic acid, neutral amino acids, γ -aminobutyric acid, and basic amino acids.³¹ An acetic acid-pyridine buffer at pH 3.4 has also been applied.³² The combination of ninhydrin followed

²⁵ Z. Peizker, *Collection Czechoslov. Chem. Commun.* **25**, 1514-16 (1960).

²⁶ E. Gründig, *Clin. Chim. Acta* **7**, 498-505 (1962).

²⁷ Darrel H. Spackman, William H. Stein, and Stanford Moore, *Anal. Chem.* **30**, 1090-1205 (1958); K. Hannig, *Clin. Chim. Acta* **4**, 51-7 (1959); G. Krampitz, *Z. Tierphysiol., Tierernähr. Futtermittelk.* **15**, 40-6 (1960); M. Dautrevaux, *Bull. Soc. Chim. Biol.* **44**, 1105-14 (1962).

²⁸ M. C. Michel, *Ann. Biol. Animale Biochim., Biophys.* **1**, 449-56 (1961).

²⁹ E. R. Cook and Mollie Lascombe, *J. Chromatog.* **3**, 75-84 (1960); S. Blackburn and G. R. Lee, *Biochem. J.* **87**, 1P-2P (1963).

³⁰ Tadataka Fukui and Masato Yasuda, *Eigo To Shokuryo* **6**, 64-7 (1953); H. E. Schendel, C. Liadsky, and H. Lenz, *S. Afr. J. Lab. Clin. Med.* **8**, 18-23 (1962).

³¹ F. Schneider, E. Reinefeld and H. Muller, *Biochem. Z.* **327**, 189-94 (1955).

³² Sadihiko Akai, Osamu Wakasa, Koe Ueki, Kenji Kuriyama, Hiroshi Sugano and Iku Watanabe, *Seitai No Kagaku* **9**, 27-33 (1958).

by copper nitrate has been applied to such samples.³³ In 0.42% sodium bicarbonate in 95% ethanol, the ninhydrin complexes are stable for 24-27 hours.³⁴

Paper chromatography for quantitative determination with ninhydrin separated the following amino acids from rye and wheat flours: aspartic acid, asparagine, glutamic acid, serine, glycine, alanine, glutamine, γ -aminobutyric acid, tyrosine, valine, leucine, isoleucine, tryptophan, and phenylalanine.³⁵

A micromethod for amino acids and peptides is based on the ultra-violet absorption of their copper salts.³⁶ The following compounds follow Beer's law in that technic: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, proline, serin, threonine, valine, alanylglycine and glycyllu-cine. Tyrosine and tryptophan do not follow Beer's law. Blank values are reduced by two-thirds by addition of water to the control.

Amino acid solutions are incubated with cupric orthophosphate in suspension. The resulting solution contains copper in proportion to the total molarity of amino acids and the copper is thereafter determined with sodium dithiocarbamate.³⁷ With polyphenols present, the results tend to be high as polyphenols complex with them.³⁸ By extraction of the copper diethyldithiocarbamate with amyl acetate, this is applicable to colored samples.³⁹ Beer's law is followed for 0.05-0.1 mg. of α -alanine per ml. Peptides and proteins do not interfere.

The copper may also be determined by cuprazone, bis-cyclohexanone-oxalyldihydrazone.⁴⁰ Interfering amino groups are destroyed by nitrous acid treatment. Compounds that interfere and are destroyed by nitrous acid treatment are: ammonium chloride, ethylenedinitrilotetraacetic acid,

³³ H. Rastetter and R. Clotten, *Ärzt. Wochschr.* **14**, 153-61 (1959); K. Lipovec, *Farm. Glasnik* **18**, 2-18 (1962).

³⁴ P. Bohley, *Naturwissenschaften* **49**, 326 (1962).

³⁵ A. N. Ponomareva and V. L. Kretovich, *Izvest. Vysshikh Ucheb. Zavedenii, Pishchevanza Tekhnol.* **1960**, No. 1, 132-4.

³⁶ J. R. Spies, *J. Biol. Chem.* **195**, 65-74 (1952); Arthur Cherkin, Howard Wolkowitz and Max S. Dunn, *Anal. Chem.* **28**, 895-6 (1956); R. G. Whitehead, *Lancet* **1964**, 250-2.

³⁷ W. A. Vincent, *Nature* **185**, 530 (1960).

³⁸ K. W. De Witt, *Chemistry and Industry*, **1955**, 1551-2.

³⁹ C. Edwin Weill and Armand Bedekian, *Microchem. J.* **1**, 89-91 (1957); cf. E. Pfeil and H. J. Goldbach, *Klin. Wochschr.* **34**, 194-5 (1956).

⁴⁰ Raymond Borchers, *Anal. Chem.* **31**, 1179-80 (1959); P. Malangeau, R. Bourdon, A. M. Nicaise and B. Masson, *Ann. Biol. Clin. (Paris)* **21**, 3-13 (1963).

glucose, hydrochloric acid, lactic acid, potassium cyanide, sodium acetate, sodium citrate, sodium hydroxide, sodium phosphate, trishydroxymethylaminomethane, and zinc sulfate. Urea, uric acid, creatine, creatinine, and ammonium ions do not interfere. Up to 25 micromole of lactic acid, glucose, galactose, sucrose, inositol, mannitol, and ammonium ions do not interfere. Polypeptides interfere. Urine must be purified with ion-exchange resin. Cystine, methionine, tryptophan, leucine, and phenylalanine complexes have a very low solubility, but this is increased by adding a known amount of glycine or aspartic acid. Histidine does not react normally. Beer's law holds up to 1.5 micromole of copper.

Amino acids are quantitatively converted to their yellow 2,4-dinitrophenyl derivatives by treatment with 2,4-dinitrofluorobenzene. The intense red color with sodium borohydride then serves for their determination.⁴¹ These derivatives may be separated by paper chromatography.

The 3-phenyl-2-thiohydantoins formed from the amino acids are separated on paper chromatograms. The spots are located with fluorescence screen and eluted with ethanol.⁴² Since cysteine and cystine do not form phenylthiohydantoin derivatives, these amino acids are oxidized with phenylisothiocyanate before coupling.

The oxidative deamination of certain α -amino acids, catalyzed by L-amino acid oxidase, produces hydrogen peroxide, which reacts with *o*-dianisidine in the presence of horse-radish peroxidase to form a colored product that has an absorption maximum at 440 m μ . The time required for the reaction to produce a small fixed amount of colored product is measured automatically and related to the initial amino acid concentration.⁴³ The concentration range is 4-50 ppm. of amino acid. A hydrolysate of protein or peptides cannot be analyzed, as the enzyme does not attack all L-amino acids.

The enzyme is applicable to the oxidation of isoleucine, α -amino-n-butyric acid, citrulline, histidine, leucine, methionine, norleucine, norvaline, phenylalanine, tryptophan, and tyrosine. Histidine and tyrosine

⁴¹ F. Sanger, *Biochem. J.* **39**, 507-15 (1945); Shiro Akabori, Ko Ohno, Tokiyi Ikenaka, Akiho Nagata and Ichiro Haruna, *Proc. Japan Acad.* **29**, 561-4 (1953); Anthony L. Levy, *Nature* **174**, 126-7 (1954); F. A. Isherwood and D. H. Cruickshank, *ibid.* **174**, 123-6 (1954); G. Zimmerman, *Chem. Tech. (Berlin)* **9**, 260-1 (1954); Gebhard Koch and Wolihard Weidel, *Hoppe-Seyler's Z. physiol. Chem.* **303**, 213-23 (1956).

⁴² John Sjöquist, *Acta Chem. Scand.* **10**, 149 (1956); *Biochem. et Biophys. Acta* **41**, 20-30 (1960).

⁴³ H. V. Malmstadt and T. P. Hadjiioannou, *Anal. Chem.* **35**, 14-16 (1963).

cannot be determined by this method because of interference due to the presence of D-configurations. Benzoic, mandelic, salicylic and iodoacetic acids, sulfonamides, aromatic sulfonic acids, aliphatic α -amino-sulfonic acids, and carbonyl reagents are inhibitors. Alanine, arginine, aspartic acid, cysteine, glutamic acid, proline, serine, threonine, and valine do not react. The accuracy of the method depends on how accurately the value for the standardized amino acid is known and the precision of the measurements.

A yellow solution is formed when amino acids treated with 2,4,6-trinitrobenzene-1-sulfonic acid is acidified.⁴⁴ The method has been applied to tyramine, histamine, glucosamine, ethanolamine, β -alanine, γ -aminobutyric acid, *p*-aminobenzoic acid, glycine, alanine, valine, leucine, isoleucine, serine, threonine, phenylalanine, tyrosine, tryptophan, cystine, methionine, histidine, lysine, arginine, aspartic acid, glutamic acid, aminocaproic acid, putrescine, cadaverine, isoamylamine, glycylglycine, glycylserine, glycylvaline, glycyltyrosine, glycyltryptophan, glycylhistidine, glycylaspartic acid, alanylglycylglycine, leucyltyrosine, alanylpargine, carnosine, and glutathione.

Paper chromatograms of amino acids are treated with xylose in the presence of sodium bisulfite. The resulting fluorescence of the spots is measured.⁴⁵

Glycine, lysine, arginine and tryptophan form azo derivatives with diazotized sulfanilic acid. Beer's law is followed at 363 $m\mu$ for glycine and lysine, and at 356 $m\mu$ for arginine.⁴⁶ Ethanol reduces extractions. The basic groups of amino acids react with sulfur dioxide, which is present partly free and in loose combination with fuchsin molecules in Schiff's reagent. As the sulfur dioxide is used up, the color of the free fuchsin molecule develops.⁴⁷

Amino acids may be determined by their colored complex dioxohydrindylidenhydrindamino cadmium salts. The ninhydrin-treated amino acids are treated with cadmium chloride.⁴⁸ The reaction with *p*-benzoquinone applied to proteins is applicable to amino acids to give a red

⁴⁴ Kazuo Satake, Tsuneo Okuyama, Mochihiko Ohashi and Tomotaka Shinoda, *J. Biochem.* **47**, 654-60 (1960).

⁴⁵ V. G. Shore and Arthur B. Pardee, *Anal. Chem.* **28**, 1479-81 (1956); cf. Bovidar Tuzina and Branko Briski, *Kem. ind. (Zagreb)* **3**, 253-6, (1954); G. A. Uzbekov, *Veprosy Med. Khim.* **4**, 64-76 (1958).

⁴⁶ Dallas Fraser and H. G. Higgins, *Nature* **172**, 459-60 (1953).

⁴⁷ S. N. Bhattacharya, *Anal. Chim. Acta* **11**, 559-62 (1954).

⁴⁸ Michel Mortreuil and Yvonne Khouvine, *Bull. soc. chim. biol.* **36**, 425-8 (1954).

coloration.⁴⁹ The phosphotungstic acid reagent is applicable to deproteinized blood or urine.⁵⁰

α -Amino acids are developed by ascorbic acid in dimethylformamide.⁵¹ By having a proper amount of water present, interference from primary amines is avoided.

Procedure—By ninhydrin. General.⁵² Mix 1 ml. of sample such as urine, serum, or cerebrospinal fluid with 1.5 ml. of 1:1 acetone-ethanol. Add 0.1 ml. of 0.5 *M* phosphate buffer for pH 6.5. Centrifuge. Add 2 ml. of a 0.5% solution of ninhydrin to the supernatant layer. Stopper, and heat at 100° for 10 minutes. Cool in ice water and dilute to 10 ml. with methanol. Filter, and read at 580 *m μ* . This detects 0.5 microgram of nitrogen. Recovery is around 99% and ammonia does not interfere.

*By hydrindantin.*⁵³ Wash Dowex 50-X4 in the hydrogen form with 1:2 hydrochloric acid in a funnel with very gentle suction. After 4-8 liters of acid, the filtrate should be colorless. Wash with water followed by a wash with 8% sodium hydroxide solution until the filtrate is alkaline. Suspend the resin in 3 times its volume of 4% sodium hydroxide solution and heat at 100° for 3 hours with occasional shaking. Decant the supernatant liquid and allow to settle for 1 hour. Replace the hot fresh 4% sodium hydroxide solution and repeat the procedure 5 times. Filter the resin, wash free from alkali, and pass through a 200-400 mesh screen with 6-8 liters of water. Filter the resin and store as the moist sodium salt.⁵⁴

Prepare a pH 3.1 buffer containing 71.4 grams of citric acid, 28.2 grams of sodium hydroxide, and 39.3 ml. of concentrated hydrochloric acid in a final volume of 3.4 liters. Prepare a pH 2.2 buffer containing 105 grams of citric acid, 42 grams of sodium hydroxide, and 80 ml. of concentrated hydrochloric acid in a final volume of 5 liters.

Prepare a pH 5.1 buffer containing 357 grams of citric acid, 73 grams of glacial acetic acid, 160 grams of sodium hydroxide, and 463 grams of sodium acetate trihydrate in a final volume of 3.4 liters. To prepare this buffer, dissolve the citric acid in 1.5 liters of water and add the sodium

⁴⁹ H. Wehle, *Pharmazie* **12**, 494-9 (1957); *ibid.* **13**, 41-4 (1958).

⁵⁰ Ilse Antener, *Schweiz. med. Wochenschr.* **81**, 970-4 (1951); *ibid.* **83**, 425-7 (1953).

⁵¹ J. Bartos, *Ann. Pharm. Franç* **22**, 383-5 (1964).

⁵² D. Miiting and E. Kaiser, *Z. physiol. Chem.* **332**, 276-81 (1963).

⁵³ Stanford Moore and William H. Stein, *J. Biol. Chem.* **211**, 907-13 (1954).

⁵⁴ Stanford Moore and William H. Stein, *J. Biol. Chem.* **192**, 663-81 (1951).

hydroxide in small portions to avoid overheating. Before use, add 5 ml. of Brij 35 solution and 5 ml. of thioglycol per liter of buffer.

Prepare the sodium acetate buffer for pH 5.5, dissolving 272 grams of sodium acetate trihydrate in 200 ml. of water with heat. Cool to room temperature, add 50 ml. of glacial acetic acid, and dilute to 500 ml. If the undiluted solution is not at pH 5.5, adjust the pH with sodium hydroxide.

Prepare chromatograph tubes having an inner diameter of 0.9 cm. and a length of 165 cm. above a sintered glass plate.⁵⁵ The sintered glass plate is sealed directly into the tube. Enclose the tubes in two connecting 750-mm. West-type condenser jackets through which water is circulated from a constant temperature bath at 30°. Wash 200 ml. of the settled sodium salt of the resin on a Büchner funnel with 1 liter of 4% sodium hydroxide solution. Place filter paper on top of the resin bed to prevent channeling. Wash with 800 ml. of the pH 5.5 buffer diluted 1:10. Stir the resin with 200 ml. of buffer to give a suspension, which, when settled, yields about equal volumes of supernatant solution and resin bed. Pour the resin into the column to fill sections 20-30 cm. in height, allow to settle, and withdraw the buffer with suction. If the rate of flow is less than 5 ml. per hour under 15 cm. pressure, resuspend the resin and remove the finest 10% from the surface of the settled bed. To the column, attach a 250 or 500 ml. separatory funnel filled with 0.8% sodium hydroxide solution containing 0.5% Brij 35 solution. Brij 35, a polyoxyethylenelauryl alcohol, is prepared by dissolving 50 grams in 100 ml. of hot water. If the detergent does not remain in solution at room temperature, increase the water to 150 ml. and use 1.35 times the detergent in the procedure as required. Attach the funnel with 3 feet of tubing, which is joined at the other end to a glass U-tube passing through a rubber micro-stopper. Pass 100 ml. of this alkaline solution through the resin overnight at 15-20 cm. pressure.

One day before using the column, replace the sodium hydroxide solution in the funnel and column with 200 ml. of buffer at pH 3.1 or 2.2. Drive the buffer through the column overnight until the pH of the effluent is the same as the buffer. For most amino acids, use a buffer at pH 3.1, with taurine and urea use a buffer at pH 2.2.

Adjust the sample solution to pH 2-2.5 and add 1-5 ml. of sample to the surface of the resin. Wash the column with three 0.3-ml. aliquots of buffer at pH 2.2. Add pH 3.1 buffer to the column above the resin and

⁵⁵ Stanford Moore and William H. Stein, *J. Biol. Chem.* **211**, 893-906 (1954).

to the separatory funnel. Mount the column over a fraction collector and circulate water at 30° through the jacket. Collect 2-ml. fractions of effluent at a maximum rate of 8 ml. per hour. After the emergence of serine (at approximately 210 ml. of effluent), increase the water temperature in the circulating jackets to 50°. At an effluent volume corresponding to twice that of the maximum of the aspartic acid peak, at approximately 210 ml. of effluent, gradually increase the ionic strength and pH of the influent to 5.1. After the emergence of histidine at approximately 700 ml., increase the temperature to 75°. The blanks are chosen from tubes before or after the peak of the amino acid. For methionine, isoleucine, and leucine, choose the blank from fractions emerging after the leucine peak. For histidine, use a blank before the emergence of the histidine peak.

To prepare hydrindantin, add with stirring a solution of 80 grams of ascorbic acid in 400 ml. of water at 40° to 80 grams of ninhydrin in 2 liters of water at 90°. Allow crystallization to proceed for 30 minutes without heating. Cool to room temperature under running water for 1 hour. Filter the hydrindantin, wash with water, and dry to a constant weight over phosphorus pentoxide in a vacuum desiccator in the dark. Store in dark glass.

As a reagent, dissolve 20 grams of ninhydrin and 3 grams of hydrindantin in 750 ml. of methyl Cellosolve. When stirring, do not incorporate air bubbles into the solution. Add 250 ml. of buffer at pH 5.5, transfer to dark bottles, and store under nitrogen. The reagent is not stable after 1 week. It is not necessary to adjust the pH of the 1-2-ml. sample effluents from the ion-exchange column, unless more than 1 ml. of 1:110 hydrochloric acid or 0.4% sodium hydroxide solution is required to bring the pH of the samples to the pH 4-6 range.

Shake the sample with 1 ml. of reagent for 10 seconds to oxidize residual hydrindantin. Heat for 15 minutes in a covered boiling water bath. Dilute with 1:1 ethanol-water, cool to below 30° with an electric fan, shake thoroughly, and read at 570 m μ for all amino acids except proline or hydroxyproline, which are read at 440 m μ .

Water supplies. For amino acids in water supplies, concentrate by passing a liter through a column of 1 \times 20 mm. of cationic KU-2 in hydrogen form. Elute the amino acids with concentrated ammonium hydroxide. Evaporate the eluate to dryness at 100°.

As reagent, dissolve 0.075 gram of cadmium chloride, 3 ml. of water, 0.6 ml. of glacial acetic acid, and 2 grams of ninhydrin in 100 ml. of

acetone. Take up the residue from the sample in 2 ml. of water and add 1 ml. of reagent. Add 5 ml. of butanol and 2 drops of saturated cadmium chloride solution. Heat at 100° for 15 minutes and separate the butanol layer. Dilute with butanol to 6 ml. and read at 530 m μ as glycine.

*By ninhydrin, in ethanol.*⁵⁶ *Urine.* To prepare the ethylenediamine-tetraacetate solution, add 9 ml. of glacial acetic acid and 2.4 grams of disodium ethylenediaminetetraacetate to 800 ml. of water. Adjust the pH to 3.7 with 4% sodium hydroxide solution and dilute to 1 liter with water. Store in a refrigerator.

To prepare the carbonate-bicarbonate buffer for pH 10.5, dissolve 21.2 grams of sodium carbonate in 1 liter of water and 16.8 grams of sodium carbonate solution with 9.5 volumes of the sodium bicarbonate solution. To prepare the glycine standard solution, dissolve 5.358 grams of glycine in 100 ml. of water. Dilute 1 ml. to 100 ml. with water to form a working standard containing 0.1 mg. of glycine nitrogen per ml. The rubber stoppers used as connections in the condensers should be soaked in 90% ethanol for 24 hours and boiled in water for 1 hour before use. They may also be wrapped in aluminum foil.

To remove ammonia, add 0.2 ml. of the carbonate-bicarbonate buffer to a 0.2-ml. sample and mix well. Immerse the tip of a small funnel in 1:16 sulfuric acid so that a drop of acid adheres to the inner wall of the stem. Wipe the outer wall and place the funnel in the sample solution, with the acid drop hanging 1-2 cm. above the liquid. Let stand 2 hours or overnight at room temperature. Remove the funnel and add 2 ml. of ethylenediaminetetraacetate solution. Prepare a blank and standards by adding 0.2 ml. of the carbonate buffer and 2 ml. of ethylenediaminetetraacetate solution to (1) 0.2 ml. of water, (2) 0.1 ml. of water, and 0.1 ml. of glycine solution, with (3) 0.2 ml. of glycine solution.

To a 0.3-ml. aliquot of the sample, standard, and blank, add 4 ml. of 0.2% ninhydrin solution in absolute ethanol. Mix well, add a boiling chip, and reflux at 100° for 20 minutes after refluxing begins. Cool, dilute to 10 ml. with 50% ethanol, and read within 1 hour at 570 m μ .

The optical density of the standards minus that of the blank is linearly proportional to the α -amino nitrogen of the standard up to 3 micrograms of α -amino nitrogen per tube. The procedure is applicable in the range of 0.6-2.5 micrograms of α -amino nitrogen. Results for urine

⁵⁶ Ayedts Khachadurian, W. Eugene Knox and Andrea M. Cullen, *J. Lab. Clin. Med.* **56**, 321-32 (1960).

are expressed as the equivalent of the micrograms of α -amino nitrogen of the standard per ml. All amino acids do not yield equal color with ninhydrin. Therefore, the value for total α -amino acids in urine depends on the individual acids present. With this method, the total color in urine samples is equivalent to approximately 80% of the total α -amino nitrogen in an average sample. Proline and asparagin gives little color with ninhydrin. Phenylalanine, tyrosine, tryptophan, taurine, threonine, arginine, and the histidines give incomplete color yield when compared with an equimolar amount of glycine by this technic.

*Heparinized plasma.*⁵⁷ To prepare the protein precipitant, add 1 part of 13.3% sodium tungstate solution to 9 parts of 1:310 sulfuric acid. Rinse 0.3 ml. of plasma into 0.7 ml. of water. Add 3 ml. of tungstic acid reagent with shaking. Centrifuge. Decant the supernatant liquid through cotton wool. Prepare a blank by adding 3 ml. of tungstic acid reagent to 1 ml. of ammonia-free water. For the standard, dissolve 35.7 mg. of monosodium glutamate in water and dilute to 100 ml. Dilute 4 ml. to 100 ml. with water for the working standard. Prepare a standard by adding 3 ml. of tungstic acid reagent to 1 ml. of working standard. To prepare the buffer, dissolve 164 grams of anhydrous sodium acetate in 1 liter of water. Dilute 120 ml. of glacial acetic acid to 1 liter with water. Add 300 ml. of the sodium acetate solution to 700 ml. of the glacial acetic acid solution and add 5 grams of disodium ethylenediaminetetraacetate and 240 mg. of potassium cyanide. Preserve with chloroform.

Dissolve 400 grams of phenol in 100 ml. of absolute ethanol by heating. Cool, add 5 grams of Permutit, and stir well for 10 minutes. Filter. Any color resulting from the oxidation of phenol does not interfere with the method. Dissolve 6 grams of ninhydrin in 100 ml. of absolute ethanol with vigorous shaking. Add 2 grams of Permutit and stir for 10 minutes. Filter, and store in a brown bottle. If the color darkens from a pale yellow-green, the reagent is contaminated.

To 0.5 ml. of a sample containing up to 0.0035 mg. of α -amino nitrogen, add 0.5 ml. of buffer and 5 ml. of phenol reagent. Shake until clear. Add 0.5 ml. of ninhydrin reagent and shake. Heat at 100° for 4-5 minutes without a stopper. Cool in water for at least 10 minutes. Shake vigorously and read after 10 minutes at 570 m μ against water. Read a blank prepared from 0.5 ml. of ammonia-free water against water.

⁵⁷ H. M. Rubinstein and J. D. Pryce, *J. Clin. Path.* 12, 80-4 (1959); cf. L. J. Fisher, S. L. Bunting and L. E. Rosenberg, *Clin. Chem.* 9, 573-81 (1963).

$$\frac{\text{O.D. test} - \text{O.D. blank}}{\text{O.D. standard} - \text{O.D. blank}} \times \frac{8}{3} \times 3.5$$

= mg. α -amino nitrogen per 100 ml. of plasma

O.D. = optical density

Cerebrospinal fluid. Rinse 0.6 ml. of cerebrospinal fluid into 0.4 ml. of water. Follow the procedure for plasma.

$$\frac{\text{O.D. test} - \text{O.D. blank}}{\text{O.D. standard} - \text{O.D. blank}} \times \frac{8}{6} \times 3.5$$

= mg. α -amino nitrogen per 100 ml. of cerebrospinal fluid

Urine. Free α -amino nitrogen. Rinse 0.2 ml. of urine into 2 ml. of 0.69% potassium carbonate solution. Place in a vacuum desiccator which contains 300-400 ml. of concentrated sulfuric acid to eliminate ammonia. Evacuate the desiccator to a pressure of 20-30 mm. mercury. Evaporate the solution to dryness overnight. Dissolve the residue in 10 ml. of water. Prepare a blank with 0.2 ml. of water and a standard with 0.2 ml. of working standard. To prepare this working standard, dilute the stock 1:1. Develop as described for the plasma sample.

$$\frac{\text{O.D. test} - \text{O.D. blank}}{\text{O.D. standard} - \text{O.D. blank}} \times 10 \times 3.5$$

= mg. of free α -amino nitrogen per 100 ml. of urine

Urine. Total α -amino nitrogen. Hydrolyze a 2-ml. sample with 1 ml. of concentrated hydrochloric acid in a sealed tube at 150° overnight. Cool, open cautiously, and remove humin by decanting the hydrolysate through cotton wool. Rinse 0.15 ml. of the hydrolysate into 2 ml. of 5.52% potassium carbonate solution. Prepare a blank with 0.2 ml. of water and a standard with 0.2 ml. of working standard as prepared for free α -amino nitrogen in urine. Add blank and standards to 2 ml. of 3.46% potassium carbonate solution. Follow the procedure for free α -amino nitrogen. Calculate 15 ml. of the hydrolysate as equivalent to 10 ml. of the sample and 0.5 ml. of urine in the final sample.

$$\frac{\text{O.D. test} - \text{O.D. blank}}{\text{O.D. standard} - \text{O.D. blank}} \times 20 \times 3.5$$

= mg. total α -amino nitrogen per 100 ml. urine

*By ninhydrin with cyanide.*⁵⁸ To prepare the acetate buffer, dissolve 2700 grams of sodium acetate in 2 liters of water. Add 500 ml. of glacial acetic acid and dilute to 7.5 liters with water. The pH should be 5.3-5.4.

To prepare the sodium cyanide solution, dissolve 0.49 gram in 1 liter of water. To prepare the acetate-cyanide reagent, dilute 20 ml. of the cyanide solution to 1 liter with the acetate buffer. To a 1-ml. sample containing 0.02-0.4 micromole of amino acid, add 0.5 ml. of cyanide-acetate reagent and 0.5 ml. of 3% ninhydrin solution in methyl Cellosolve. Heat for 15 minutes at 100° and rapidly add 5 ml. of a 1:1 isopropanol-water mixture. Shake vigorously, cool to room temperature, and read at 570 m μ . Read proline and hydroxyproline at 440 m μ . The samples, when heated, turn bright red. When cooled and mixed with isopropanol, the color turns purple, with the exception of proline and hydroxyproline, which turn yellow.

Alternative technic. To prepare the citrate buffer at pH 5, dissolve 21 grams of citric acid in 200 ml. of water, add 200 ml. of 4% sodium hydroxide solution, and dilute to 500 ml. Store in the cold with thymol. Dissolve 0.1628 gram of potassium cyanide in water and dilute to 250 ml. Dilute 5 ml. of potassium cyanide solution to 250 ml. with methyl Cellosolve. Mix 50 ml. of 5% ninhydrin solution in methyl Cellosolve with 250 ml. of the potassium cyanide solution in methyl Cellosolve. Store overnight before use. The solutions turn from red to yellow. Mix 0.5 ml. of the citrate buffer with 1 ml. of a sample containing 0.000005-0.0056 mg. of amino nitrogen. Add 1.2 ml. of the potassium cyanide-methyl Cellosolve-ninhydrin reagent. Mix well and heat for 15 minutes at 100°. Cool for 5 minutes under running water. Dilute to a convenient volume with 60% ethanol, shake well, and read at 570 m μ for all amino acids except proline and hydroxyproline, which are read at 440 m μ . Read against 1.5 ml. of citrate buffer and 1.2 ml. of ninhydrin reagent carried through the entire procedure.

*By ninhydrin. Two solvent chromatography.*⁵⁹ *Brain tissue.* Homogenize 5 samples in 7.5 times their weight of ice water. Mix 65 ml. of the homogenate with 65 ml. of saturated aqueous picric acid. Stir with 5 grams of acid-washed Celite and remove the precipitate by filtering. Wash the filter cake with 50 ml. of water. Stir the filtrate for 30 minutes at room temperature with 25 grams of 50-100 mesh moist Dowex 2-XS

⁵⁸ Hyman Rosen, *Arch. Biochem. Biophys.* **67**, 10-15 (1957).

⁵⁹ R. S. De Ropp and E. H. Snedeker, *Anal. Biochem.* **1**, 424-32 (1960).

resin in the chloride form. Pour on a coarse, sintered glass filter and wash with three 20-ml. portions of 0.1% acetic acid solution. Combine the filtrate and washings, shell-freeze, and lyophilize. Dissolve the dry residue in 4 ml. of water to give a solution equivalent to 2.0375 gram of brain tissue per ml.

Alternatively, homogenize 5 samples in three times their total weight of cold 80% methanol. Centrifuge 30 ml. of the homogenate at 2000 g for 15 minutes. Adjust 20 ml. of the supernatant liquid to pH 4 with 1:10 hydrochloric acid and concentrate *in vacuo* to 5 ml. Allow to stand in the cold overnight, and remove any precipitate by centrifuging.

Rule $18\frac{1}{4} \times 22\frac{1}{2}$ -inch sheets of Whatman No. 1 filter paper into five sections 11.5 cm. wide. Apply the sample as a streak 7 cm. long at a distance of 8 cm. from the upper long edge of the paper. The sample is approximately 2-4 ml.

Solvent system 1 consists of 5 parts of *n*-butanol, 1 part of glacial acetic acid, and 4 parts of water. Use solvent system 1 alone when determining glutathione, phosphoethanolamine, glutamic acid, alanine, ethanolamine, and γ -amino-butyric acid only.

Solvent system 2 consists of 40 parts of methylethylketone, 40 parts of *tert*-butanol, 20 parts of water, and 1 part of pyridine. Run the sheets in system 1 three consecutive times for 16 hours each, and dry for 8 hours between runs. Run sheets in system 2 twice for 16 hours each, and dry 8 hours between runs.

Develop a guide strip for alanine with ninhydrin and develop the sheets with system 1 until the solvent front just reaches the point where the alanine should be indicated by the guide strip. Dry the sheets in a moving air stream for 2 hours. Spray with pyridine and heat for 15 minutes at 60°. Pass rapidly through 0.25% ninhydrin solution in acetone and heat for 25 minutes at 60°. Cut the chromatograms into strips corresponding to the separated amino acids. Cut each strip into pieces and elute with a solution containing 75% of acetone adjusted to pH 8.5 with 1 ml. per liter of *M* phosphate buffer. The amount of eluting solution depends on the intensity of the ninhydrin color. The usual amount is 4-12 ml. Stopper, shake periodically, decant, and read at 570 m μ .

The first run with system 1 gives satisfactory separation of glutathione, phosphoethanolamine, glutamic acid, alanine, ethanolamine, and γ -amino butyric acid. Glutamine and taurine are not separated. Aspartic acid and glycine are partially separated. The second run with system 1 (after the alanine location) separates taurine and ethanolamine, glutamine and glutamic acid, aspartic acid and phosphoethanolamine, γ -amino-

butyric acid and glycine. The alcohol separation of the sample gives low values for glutathione and phosphoethanolamine.

*By ninhydrin. Separation of amino acids by electrophoresis.*⁶⁰ *Serum.* To prepare Zeocarb 225H, wash 400 grams of 60-80 mesh resin with 1 liter of 1:3 ammonium hydroxide followed by 5 liters of water. Treat with 100 ml. of 2:3 hydrochloric acid and 300 ml. of 1:4 hydrochloric acid, and wash with water until the washings are free from chloride. Store the resin under water until use.

A column for the resin measures 5 cm. with a 0.4-cm. diameter, a 1-mm. constriction at the bottom, and a 20-ml. bulb reservoir at the top. Place a glass wool plug above the constriction and pour an aqueous slurry of the resin into the column. Slowly pass 1.5 ml. of fresh serum through the column. Wash with 50 ml. of water to remove proteins, sugars, acids, and other compounds. Elute the amino acids with 20 ml. of 1:2 ammonium hydroxide. Evaporate the eluate under reduced pressure in a vacuum desiccator containing sodium hydroxide pellets and fused calcium chloride. Dissolve the residue in 5 ml. of warm water and evaporate the water from a 4-ml. portion under reduced pressure over sodium hydroxide pellets. Partition the extract into groups of amino acids by electrophoresis in 6% acetic acid on Whatman 3 paper in a conventional type of horizontal apparatus (Fig. 6).

Transfer the extract to a strip of paper 5 cm. wide and 20 inches long, so that there is even distribution along the load line. Do not allow the amino acids to run along the edge of the paper.

Dissolve the residue in 0.05 ml. of warm water and evenly distribute it over an area 4 cm. \times 3 cm. adjacent to the load line, which is 2.5 inches from the anode end of the strip. Allow this extract to partially dry. Rinse the tube with 0.05 ml. of water, transfer to the paper, and dry with cold air. Clamp the strip into the electrophoresis apparatus and wet evenly with 1:16 acetic acid from both ends so that the advancing liquid fronts meet at the loading line. Carry out the electrophoresis for 2.5-3 hours at 1350 volts with a current passing at $2\frac{1}{2}$ milliamperes per strip of paper 5 cm. wide. Dry the paper *in situ* with a stream of cold air.

To minimize electro-osmotic flow of the buffer solution, substitute for the customary wet anode a 26 SWG platinum wire between two methylmethacrylate strips, which are pressed together by elastic bands. When in operation, the end of the paper is folded and nipped between

⁶⁰ E. R. Cook and Mollie Lascombe, *J. Chromatog.* **3**, 75-84 (1960).

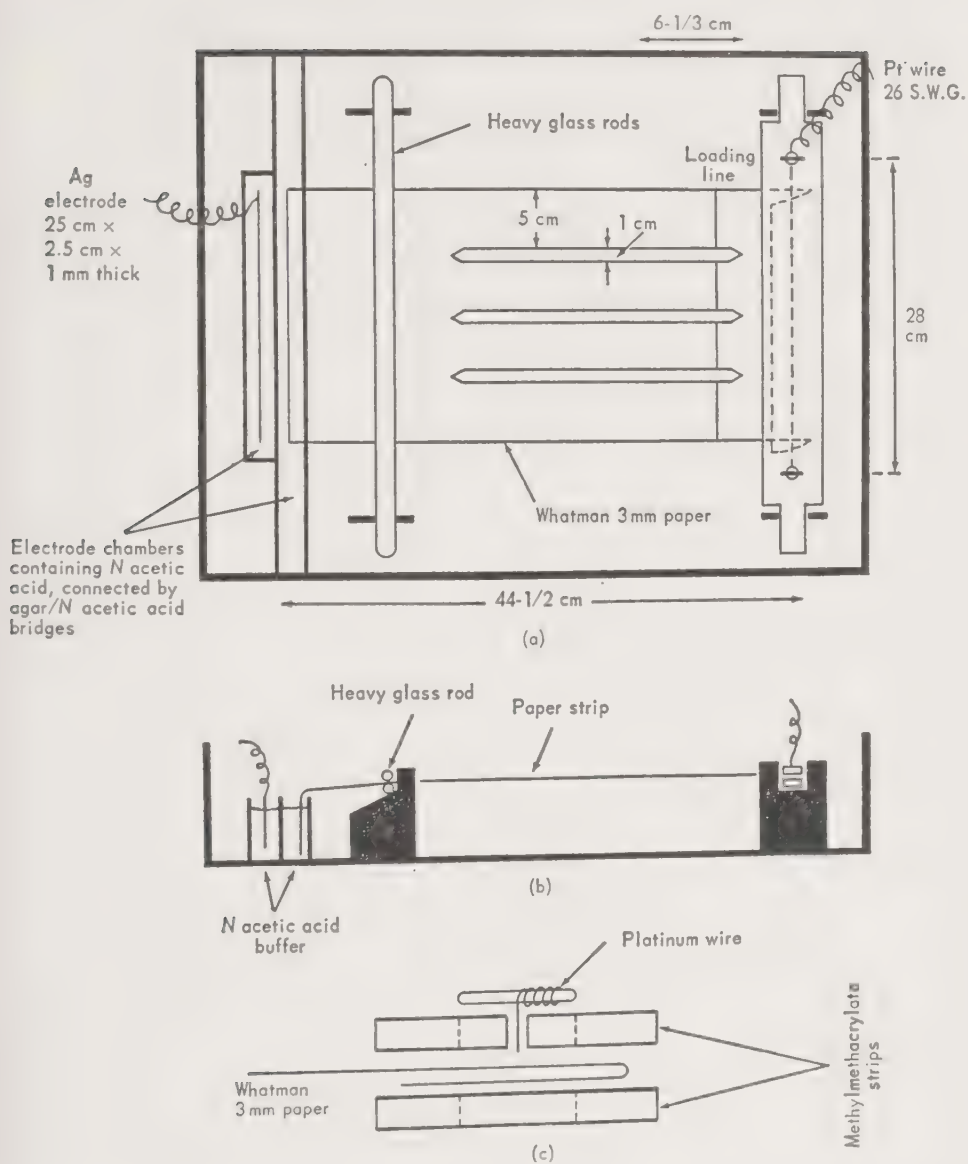


FIG. 6.

Layout of the apparatus and paper for electrophoresis. (a) The apparatus, drawn to scale, is home-built from methylmethacrylate sheets. The thick paper is held at one end between two wide methylmethacrylate strips (b), which are supported in two methylmethacrylate holders, slotted vertically and clamped at the other end between two heavy glass rods, resting on a methylmethacrylate incline. This system holds the paper taut, and pools of electrolyte cannot accumulate. The liquid compartments are filled with 1:16 acetic acid and connected by two agar 1:16 acetic acid bridges not shown in the plan; (c) an expanded plan of the platinum wire electrode

the methylmethacrylate strips to force the wire onto the set paper surface and ensure good electrical contact.

Immerse the paper strips in acetone containing 0.1% of *o*-coumaric acid and dry at room temperature for 4-5 minutes. Heat in an oven at 100° for 90-120 minutes. Expose the paper to ultraviolet light from a Mazda MBW/U bulb, which produces yellow fluorescent bands of the amino acid/coumaric acid complexes. Imino acids, proline, and hydroxyproline are also visible. The most rapidly moving band contains ornithine, lysine, histidine, and arginine. If it is diffuse and difficult to see, concentrate by ascending chromatography with water followed by drying and relocating with the ultraviolet technic.

Cut out the bands, drop into test tubes, and extract with 5 ml. of water for at least 2 hours with occasional shaking. Evaporate a 3-4 ml. portion of the extract under suction in a desiccator over sodium hydroxide pellets. Dissolve the residue in 0.02 ml. of water and apply as a thin streak 2 cm. long to a sheet of Whatman No. 1 filter paper 22½ inches long by 8 inches wide. Dry with cold air. Wash out the test tube with two 0.02-ml. portions of water. Hang the sheet in a glass tank and place in the dark. Separate the amino acids by descending chromatography with a mixture of 60 ml. of butanol, 15 ml. of ethanol, 10 ml. of ethyl acetate, 20 ml. of water, and 2 ml. of glacial acetic acid.

After 24 hours, remove the sheet and dry in a stream of cold air. Spray evenly with a mixture of 100 ml. of 95% ethanol, 2 ml. of lactic acid, and 2 grams of ninhydrin. Air dry for 10 minutes at room temperature in a gentle air current. Heat at 105° in an oven for 4 minutes. Cut out the colored spots containing serine, valine, leucine, and isoleucine, and place in tubes containing 7.8 ml. of 90% ethanol and 0.2 ml. of 1% cadmium sulfate solution. Let stand at room temperature for 10-20 minutes. Read the pink solutions at 520 m μ .

After 40 hours, remove similarly prepared sheets and follow the above procedure. Band 1 contains aspartic acid and hydroxyproline. Band 2 contains cysteine, aspartic acid-amine, glutamic acid-amine, citrulline, glutamic acid, threonine, proline, tyrosine. Band 3, previously eluted, contains serine, valine, leucine, and isoleucine. Band 4 contains alanine. Band 5 contains glycine. Band 6 which is removed after 72-96 hours contains ornithine, lysine, histidine, and arginine. The solvent for ornithine, lysine, histidine, and arginine is the same as the other with the exception that 40 ml. of butanol and 20 ml. of glacial acetic acid are used.

Develop aliquots of 0.0025-0.05 mg. of leucine simultaneously with

the samples in the paper chromatography procedure to obtain a straight-line graph. Calculate the individual amino acids in terms of leucine.

Small quantities of proline or hydroxyproline are not measured. To estimate the proline, cut out the yellow proline spot obtained by the procedure and place it in 8 ml. of a solution consisting of 2.5 ml. of 20% calcium chloride solution and 97.5 ml. of glacial acetic acid. Heat at 100° for 5 minutes. Cool rapidly and read at 520 m μ . The pink color is stable for 15 minutes in daylight and 1 hour in the dark. Beer's law is followed up to 0.045 mg. of proline.

Since the movement of the amino acids during electrophoresis depends on the complexity of the mixture, run duplicate aliquots of serum extract containing known amino acids along with the sample.

*By ninhydrin. Alcohol molasses residues.*⁶¹ Mix a 1-gram sample with 25 ml. of 1:4 sulfuric acid and hydrolyze at 100° for 5 hours. Dilute to about 75 ml. and adjust to pH 2-3 with barium hydroxide. Filter off the barium sulfate. Pass the filtrate through an 8 \times 1-cm. column of KU-2 resin in the hydrogen form at a rate of about 0.3 ml. per minute. Elute the amino acids with 1:7 ammonium hydroxide.

Evaporate the eluate to dryness *in vacuo* at 50-60°. Take up the residue in 1-2 ml. of 1:10 hydrochloric acid. Separate the acids in an aliquot of this solution by two-dimensional paper chromatography. The first solvent is butanol-acetic acid-water at 4:1:5. It is applied at room temperature for 24 hours with the solvent replaced twice. The second solvent similarly applied is butanol-phenol-acetic acid-water at 5:5:2:10.

Dry the chromatogram at room temperature for 12 hours. Treat with ninhydrin in 4% acetic acid and dry at 80° for 6 minutes. Elute the individual spots with 50% propanol or isopropanol at room temperature. Make the solution 0.05% with copper sulfate and read at 530 m μ .

*By cupric chloride.*⁶² The sodium borate buffer is a 0.191% solution of powdered sodium borate decahydrate in water. Add 6 grams of sodium chloride to 100 ml. of the borate buffer for use.

To 5 ml. of a buffer solution, add 5 ml. of an aqueous dilution of the sample. Add 0.1 ml. of 0.852% cupric chloride dihydrate solution and shake. After 5 minutes, centrifuge at 3000 rpm. for 20 minutes. As a

⁶¹ E. A. Kozlov, G. N. Shevchuk and S. B. Serebryanyi, *Ukr. Khim. Zhur.* 29, 453-8 (1963).

⁶² W. A. Schroeder, Lois M. Kay and Rene S. Mills, *Anal. Chem.* 22, 760-3 (1950).

blank, add 5 ml. of sample to 5 ml. of buffer and add 0.1 ml. of water to compensate for the cupric chloride in the reaction mixture. Read at 235 m μ .

*As copper by sodium diethyl dithiocarbamate.*⁶³ Incubate a 0.1-ml. sample overnight with 3 ml. of 1% cupric orthophosphate suspension and 2 ml. of British Drug Houses Universal buffer solution at pH 7. Centrifuge, and mix 0.1 ml. of the supernatant liquid with 3 ml. of buffer at pH 7 and 0.1 ml. of 1% sodium diethyl dithiocarbamate solution. Read at 440 m μ after 15 minutes. If the color is too dense to read or if precipitation occurs, dilute the supernatant liquid before mixing with the reagent.

*As copper by cuprizone.*⁶⁴ Dissolve 13.7 grams of tribasic sodium phosphate dodecahydrate in 200 ml. of water and filter. Dissolve 2.8 grams of cupric chloride dihydrate in 100 ml. of water. To prepare the washed copper phosphate, add 20 ml. of the cupric chloride solution to 40 ml. of the sodium phosphate solution with swirling. Centrifuge the suspension for 5 minutes. Remove the supernatant liquid and replace with an equal volume of 0.191% borax buffer. Resuspend the copper phosphate, centrifuge, and repeat the washing. Finally, suspend the copper phosphate in 100 ml. of borax buffer and add 6 grams of solid sodium chloride.⁶⁵ Age 2 days before use. To prepare the reagent, dissolve 200 mg. of cuprizone, bis(cyclohexanoneoxalyl)dihydrazone, in 100 ml. of 50% ethanol with gentle heating. Add a 0.2-ml. blood sample to 2 ml. of 5% trichloroacetic acid solution, mix, and centrifuge. Neutralize 0.5 ml. of the supernatant liquid with 0.5 ml. of 1% sodium hydroxide solution and add 1 ml. of the copper phosphate suspension. For other samples, add 1 ml. of copper phosphate suspension directly to 1 ml. of sample containing 0.1-1 micromole of amino acid.

Neutralize, if necessary, with 4% sodium hydroxide solution to the faint pink color of phenolphthalein indicator. Mix, and let stand for 5 minutes. Centrifuge at 2000 rpm. for 5 minutes. To 1 ml. of the supernatant liquid, add 5 ml. of 0.6% boric acid solution and 0.2 ml. of cuprizone reagent. Read after 5 minutes at 600 m μ . Calculate 1 micromole of copper equivalent to 2 micromoles of amino acid or 28 mg. of amino nitrogen.

⁶³ W. A. Vincent, *Nature* **185**, 530 (1960).

⁶⁴ Raymond Borchers, *Anal. Chem.* **31**, 1179-80 (1959).

⁶⁵ W. A. Schroeder, Lois M. Kay and Rene S. Mills, *ibid.* **22**, 760-3 (1950)

In the presence of interferences. Heat 1 ml. of the sample solution with 0.5 ml. of 7% sodium nitrite solution and 0.5 ml. of 1:16 acetic acid solution at 100° for 10 minutes. Cool and neutralize with 0.5 ml. of 4% sodium hydroxide solution. Add 2.5 ml. of copper phosphate suspension and follow the procedure from "Neutralize if necessary with . . ."

*As 2,4-dinitrophenyl derivatives.*⁶⁶ Prepare two solvent systems. For a single phase for mono-amino mono-carboxylic acids, use 80 parts of *n*-propanol and 20 parts of water. For dicarboxylic amino acids, use 60 parts of *n*-propionic acid, 30 parts of *tert*-butanol, and 10 parts of water.

Spot amino acid solutions containing 0.02-0.001 *M* of amino acid at 2.5 cm. intervals along the starting line of filter paper with a micropipet. Suspend the sheets in a glass trough without solvent, which is supported in an accumulator jar. Add solvent to the bottom of the jar. Close and seal.⁶⁷

Remove the solvent from the chromatogram by hanging the paper in an air current for 3-4 hours. With the *n*-propionic acid, steam the paper for a short time after the treatment with a current of air to remove the last traces of the acid. Wash the paper with 12% acetic acid solution, water, and 35% ammonium hydroxide solution to remove impurities. Spray the paper at the rate of 5 ml. per 100 cm. with a dinitrofluorobenzene reagent in alcohol containing borate buffer. The reagent for monocarboxylic acids contains 0.06 gram of dinitrofluorobenzene, 33 ml. of ethanol, and 17 ml. of 0.2 *M* borate buffer at pH 8.4. With dicarboxylic acids, a 0.2 *M* borate buffer at pH is substituted.

After spraying, heat the papers in a closed water-jacketed oven at 80° for 30 minutes, or leave the papers overnight in a dark room at room temperature. If the amount of amino acids is very small, bleach the yellow background color by exposure to hydrogen chloride gas. The dinitrophenyl amino acids remain orange-yellow.

Cut the paper containing the dinitrophenyl amino acid from the chromatogram and elute with a solution containing 5 ml. of 0.2 *M* borate buffer at pH 8.4 and 10 ml. of 95% ethanol. Collect 0.5 ml. of the extract. Evaporate the extract to dryness by blowing a stream of cold air into the test tube. To the residue, add 1 ml. of 91% sulfuric acid. Shake to dissolve dinitrophenyl amino acid. Shake with 3 ml. of benzene for 3 minutes to extract dinitrophenol. Let stand for 5 minutes and aspirate the benzene layer. Repeat the extraction 5 times.

⁶⁶ Anthony L. Levy, *Nature* **174**, 126-7 (1954).

⁶⁷ C. S. Hanes and F. A. Isherwood, *ibid.* **164**, 1107-12 (1949).

Dilute the sulfuric acid layer to 30% by adding 2 grams of crushed ice. Immerse the tube in an ice-alcohol bath and shake until the ice is melted. Extract the dinitrophenyl amino acid by shaking with 3 ml. of 10% *tert*-amyl alcohol solution in benzene and let stand for 5 minutes. Use 5% ethanol solution for phenylalanine. Aspirate the clear upper layer and repeat the extraction twice. Shake the combined extracts with 1 ml. of a saturated sodium bicarbonate solution and let stand. Read the aqueous layer at 365 $m\mu$ or 404 $m\mu$ against a reagent blank.

For glutamic acid, follow the above procedure, ending with "Evaporate the extract to dryness by blowing a stream of cold air into the test tube." Dissolve the dried alcoholic extract in 1 ml. of 91% sulfuric acid, which has been cooled to -12° in an ice-alcohol bath. Extract as above with 3 ml. of 30% toluene solution in benzene, keeping the test tube in an ice-alcohol bath. Repeat the extraction 5 times. To the acid layer, add 2 grams of crushed ice and shake while still in ice-alcohol bath until the ice is melted. Follow the above procedure, starting with "Extract the dinitrophenyl amino acid . . ."

Alternatively, stir a 3-ml. sample containing 20-30 micromoles of amino acid with a slight excess of 1-fluoro-2,4-dinitrobenzene for 80

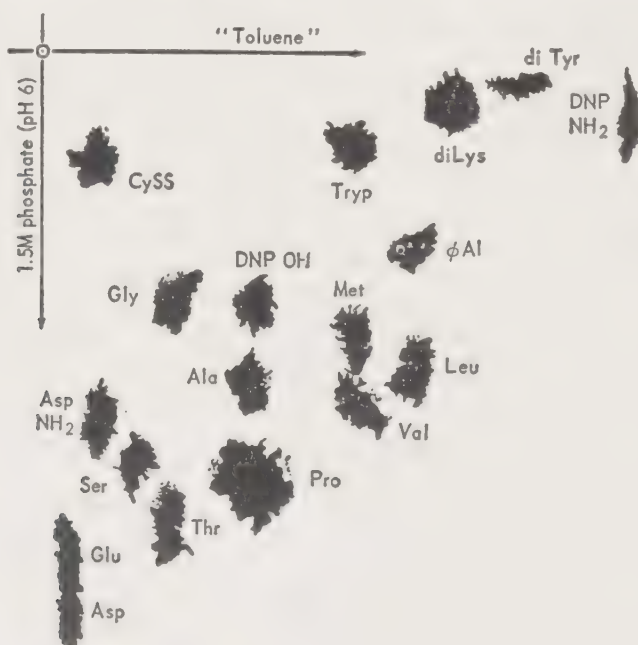


FIG. 7.

2-Dimensional chromatogram of a synthetic mixture of dinitrophenyl amino acids (approx. 0.02 μ M of each)

minutes at 40° and pH 9. Maintain the pH by intermittent addition of alkali. Extract excess fluorodinitrobenzene with ether. Acidify the solution and extract the dinitrophenyl amino acids with five 5-ml. portions of ether. The aqueous solution contains dinitrophenyl arginine and α -dinitrophenyl histidine. Dilute the aqueous solution to 10 ml. Apply a 2-ml. aliquot of the ether solution and a 1-ml. aliquot of the aqueous solution to adjacent corners of filter paper, $18\frac{1}{4} \times 22\frac{1}{2}$ inch. Irrigate the paper by the ascending procedure with a 5:3:1.5:3 mixture of toluene-chloroethanol-pyridine-1:25 ammonium hydroxide. Dry the chromatogram for 3-4 hours at 40° and cut out the spots of dinitrophenylarginine and α -dinitrophenylhistidine. Run the paper in the second dimension by the descending procedure with a 1.5 M phosphate buffer prepared with 13.8% monobasic sodium phosphate solution and 7.1% dibasic sodium phosphate solution. (See Fig. 7.) Cut out the spots and three blanks from the sheet. Add 4 ml. of water to each spot in a tube and heat at 55-60° for 15 minutes to completely elute the color. Cool to room temperature for approximately 15 minutes and decant. Read at 360 m μ for all amino acids except dinitrophenyl proline, which is read at 385 m μ , against a water blank. Correct for blank readings.

*As phenylthiohydantoin derivatives.*⁶⁸ To prepare the buffer, dilute 2 ml. of 1:8 acetic acid and 1.2 ml. of triethylamine to 25 ml. with water. Mix with an equal volume of acetone. The pH is 10.1. Generate hydrogen chloride by pouring sulfuric acid into concentrated hydrochloric acid. Wash the gas by passage through concentrated sulfuric acid and thoroughly saturate acetic acid with hydrogen chloride at room temperature.

Dissolve 5 micromoles of amino acids in 25 ml. of buffer. Add 25 ml. of a solution containing 0.6 ml. of phenyl isothiocyanate per ml. of acetone. Gently shake, stopper, and heat at 25° for 2.5 hours. Place in a desiccator over phosphorus pentoxide and carefully evaporate for 15 minutes using a water pump. Evaporate overnight with an oil pump giving a vacuum of 0.001 mm. of mercury.

Dissolve the residue in 10 ml. of water and add 20 ml. of acetic acid saturated with hydrogen chloride. Shake gently, stopper, and heat at 25° for 6 hours to convert the phenylthiocarbamyl derivatives to phenylthiohydantoin derivatives of the amino acids. Evaporate the solvent in a desiccator over potassium hydroxide as described above.

For proteins and peptides, add 0.3 ml. of 1:1.5 hydrochloric acid to 0.5-1 mg. of sample in a quartz tube 150 mm. long with an inner diameter

⁶⁸ John Sjöquist, *Biochim. et Biophys. Acta* **41**, 20-30 (1960).

of 8-10 mm. Fill the tube with nitrogen gas, seal, and maintain at 110° for 22 hours. Cool, open, and evaporate to dryness in a desiccator *in vacuo* over potassium hydroxide. Remove any remaining hydrochloric acid by repeated evaporations after adding water. Add 25 ml. of buffer and 25 ml. of acetone-phenyl isothiocyanate solution to the dry residue and follow the above procedure, starting with "Gently shake, stopper and heat . . ."

For cysteine and cystine samples, mix 1 ml. of 30% hydrogen peroxide solution and 9 ml. of 99% formic acid and let stand for 2 hours at 25°. Dissolve 1 mg. of protein in 0.2 ml. of 99% formic acid and add 0.2 ml. of peroxide-formic acid reagent. Mix, oxidize at 25° for 2 hours and evaporate the solvent to dryness in a desiccator *in vacuo* over potassium hydroxide pellets. Add water and remove any remaining acid by evaporation. Follow the above procedure, starting at "For proteins and peptides . . ."

The solvents for the separation of the 3-phenyl-2-thiohydantoin derivatives are as follows:

- Solvent 1: 60 heptane, 5 ethylenechloride, 5 propionic acid, 5 of 75% formic acid
Solvent 2: 60 heptane, 30 ethylenechloride, 5 of 75% formic acid
Solvent 3: 30 heptane, 60 ethylenechloride, 5 of 75% formic acid
Solvent 4: 40 heptane, 30 *n*-butanol, 9 of 75% formic acid

Solvents 1, 2, and 3 give two phases each when prepared. Discard the lower phase. Mix the solvents before use, especially solvent 4 where esterification occurs between butanol and formic acid.

Prepare 4 glass tanks, 20 × 30 × 60 cm. high. Equilibrate as follows: (1) equilibrate for the moving phase and 90% formic acid as the stationary phase; (2) equilibrate for the moving phase and 90% formic acid as the stationary phase; (3) equilibrate for the moving phase and 75% formic acid as the stationary phase; (4) 25% formic acid as the stationary phase. To prepare the chromatography paper, soak in 0.1% disodium ethylenediaminetetraacetate solution. Dry at 100° for 30-60 minutes. Apply the sample solution to the central strip, the sample and blank to the right and left. If a volume larger than 1 ml. is applied, dry with a stream of nitrogen gas. Prepare four papers, one for each solvent system for each amino acid determination. Hang the papers in the chromatography jars and allow to equilibrate for 15 minutes in jars 1, 2 and 3 and for 30 minutes in jar 4. Add the moving phase and allow the

papers to develop for 2.5-3.5 hours. Dry papers 1, 2 and 4 at 100° for 30 minutes, and paper 3, for 1.5 hours. Locate the spots with an ultraviolet light by means of a fluorescent screen.

To prepare the fluorescent screen, spray evenly a sheet of transparent Plexiglas with a suspension of fluorescent zinc silicate in chloroform. Spray a hot solution of Plexiglas chips in isoamylacetate as a protective coating. Paste black strips to the screen to correspond to the cut out strips of the chromatogram to facilitate locating the spots. Mark the spots and cut out with an approximate margin of 3 mm. Elute with 2 ml. of 95% ethanol for 1 hour at 40°. Read at 269 m μ , except for the serine and threonine derivatives, which are read at 320 m μ .

In chromatographic system 1, leucine and isoleucine travel as one spot. They are therefore determined together. On chromatogram 1, proline travels with phenylalanine. On chromatogram 2, proline moves with valine. Since the valine and phenylalanine derivatives can be determined separately in these chromatograms, the proline is estimated by subtracting their respective extinction values from the sum of the proline plus valine or from the sum of the proline plus phenylalanine. The lysine derivative is determined similarly. On chromatogram 2, the lysine travels with phenylthiourea or with hydroxyproline and glycine. On chromatogram 3, it travels with alanine and tryptophan. Phenylthiourea and glycine derivatives are measured without interference on chromatogram 3; alanine and tryptophan derivatives are measured without interference on chromatogram 2. Lysine can be determined by subtraction. Since tryptophan is destroyed by hydrolysis in hydrochloric acid, the amount of this amino acid is estimated by other methods.

*As peroxide by o-anisidine.*⁶⁹ To prepare the phosphate buffer at pH 7, dissolve 1.36 grams of monobasic potassium phosphate in 900 ml. of deionized water, adjust to pH 7 with 4% sodium hydroxide solution, and dilute to 1 liter. Add 250 ml. of phosphate buffer to 12 mg. of horse-radish peroxidase and dissolve. Add 2.5 ml. of absolute methanol containing 10 mg. of *o*-dianisidine per ml. Add 250 ml. of phosphate buffer and bubble oxygen through the reagent for 5 minutes. This is designated reagent A. As reagent B, dissolve 0.15 gram of potassium chloride in 200 ml. of phosphate buffer.

To prepare the enzyme solution, dissolve L-amino acid oxidase in reagent B just before use, filter, and store in ice during use. The amount

⁶⁹ H. V. Malmstadt and T. P. Hadjiioannou, *Anal. Chem.* **35**, 1416 (1963).

of enzyme to be added depends on the amino acid to be determined and the source of the enzyme. Prepare a trial solution of the enzyme and adjust the concentration so that measurement readouts of not less than 15-20 seconds up to a few minutes are obtained for the analysis of the most concentrated and the most dilute amino acid solutions. Dilute the amino acids to the concentration ranges shown in Table 15.

Use the Sargent Model O-RR automatic reaction rate adapter in combination with the Model O comparator as control unit. Switch the Sargent Spectro-Electro unit to the spectro position about 1 hour before the measurements are started. Dial the 650 position on the filter wheel and place the Corning No. 5030 filter in the auxiliary holder. Throw the polarity switch to position 1. Turn the Model A comparator unit to ON and the selector switch to $\text{PNP} \pm 0.01$ volt. Set the comparator Zero Adjust control at 5.25. Draw 0.1 ml. of L-amino acid oxidase into a 0.1-ml. micro-syringe or 0.25 ml. into a 0.25 ml-microsyringe for isoleucine, citrulline, or α -amino-*n*-butyric acid, and allow to remain at room temperature. To 3 ml. of reagent A, add 1 ml. of sample. Throw the comparator reagent selector switch to position 1 to start the stirring and adjust the Spectro balance control so that the motor needle is at the center. Set the comparator zero adjust at 4.50, inject the amino acid oxidase into the sample, and press at once the start button on the Model O-RR adapter. The analysis is completed automatically and the number on the readout dial is recorded. Repeat the balance step every 10-15 minutes to compensate for any drift.⁷⁰

*By 1-fluoro-2,4-dinitrobenzene.*⁷¹ *Serum.* As a 0.1 M buffer, dissolve 38.1 grams of sodium tetraborate pentahydrate in about 750 ml. of water. Adjust to pH 10.0 by addition of 50% sodium hydroxide solution and dilute to 1 liter. As a stock reagent, dissolve 0.65 ml. of 1-fluoro-2,4-dinitrobenzene in 50 ml. of acetone. This is stable for 3 months under refrigeration. Immediately before use, mix 0.1 ml. of the stock reagent with 1 ml. of buffer.

Mix 1 ml. of serum, 8 ml. of 1:2 sulfuric acid, and 1 ml. of 10% sodium tungstate solution. Filter. To 1 ml. of filtrate, add 1 ml. of the buffer and 0.1 ml. of the diluted reagent. Heat at 56° for 15 minutes. Add 1.1 ml. of 1:10 hydrochloric acid and mix. Read at 420 m μ against water.

⁷⁰ For further discussions of the apparatus, see: H. V. Mahstadt and S. I. Hedjioannov, *ibid.* **34**, 452-5 (1962).

⁷¹ R. D. Rapp, *Clin. Chem.* **9**, 27-30 (1963).

TABLE 15. AUTOMATIC REACTION-RATE RESULTS FOR
AQUEOUS SOLUTIONS OF AMINO ACIDS

<i>Amino acid</i>	<i>Concentration</i> $M \times 10^4$	<i>Amino acid in</i> <i>4.1 ml. sample,^a μg.</i>		<i>Relative</i> <i>error</i>
		<i>Taken</i>	<i>Found</i>	
L- α -amino-n-butyric acid	7.5-25	65.0	65.0	...
		130.0	130.0	...
		130.0	131.0	+0.8
L-Citrulline	2-10	70.1	69.7	-0.6
		70.1	70.8	+1.0
DL-Citrulline		105.1	104.1	-1.0
L-Isoleucine	2.5-10	27.8	27.6	-0.6
		83.4	81.8	-1.9
DL-Isoleucine		27.8	28.3	+1.7
		83.4	85.4	+2.4
L-Leucine	2.5-6	39.4	39.8	+1.0
		39.4	38.7	-1.8
		78.7	77.8	-1.1
DL-Leucine		39.4	39.4	...
		39.4	40.4	+2.5
		78.7	78.7	...
L-Methionine	1.5-3	29.8	29.6	-0.7
		29.8	30.4	+2.0
DL-Methionine		44.8	45.2	+0.9
L-Norleucine	2-4	39.4	39.4	...
		39.4	39.2	-0.5
DL-Norleucine (synthetic)		39.4	39.2	-0.5
L-Norvaline	6-15	70.3	68.3	-2.8
		70.3	70.9	+0.9
		140.6	138.8	-1.3
DL-Norvaline		140.6	140.1	-0.4
		117.1	113.8	-2.8
L-Phenylalanine	1-2	16.5	16.5	...
		16.5	16.3	-1.2
		33.0	32.8	-0.6
DL-Phenylalanine		33.0	33.6	+1.8
L-Tryptophan	1.5-4	30.6	30.1	-1.6
		61.3	60.6	-1.1
		61.3	62.6	+2.1
DL-Tryptophan		30.6	30.9	+1.0
		61.3	62.0	+1.1
		61.3	61.3	...

^a Concentrations of DL-mixtures refer to that of the L-isomer.

*By 2,4,6-trinitrobenzene-1-sulfonic acid.*⁷² Mix 1 ml. of sample containing 0.01-0.8 micromole of amino acid with 1 ml. of 4% sodium bicarbonate solution and 1 ml. of 0.1% 2,4,6-trinitrobenzene 1-sulfonic acid solution, and place in the dark for 2 hours at 40°. Add 3 ml. of the resulting orange solution to 1 ml. of 1:10 hydrochloric acid and read at 340 m μ against a reagent blank.

*By fluorescence.*⁷³ Prepare two solvent systems. Solvent 1 consists of 4 parts of butanol, 1 part of glacial acetic acid, and 5 parts of water. To prepare the mobile phase, mix 25 grams of phenol, 25 grams of *m*-cresol, and 7 ml. of borate buffer at pH 9.3. The borate buffer contains 200 ml. of 0.62% boric acid solution and 113.5 ml. of 0.4% sodium hydroxide solution. As solvent 2, mix 250 ml. of buffer with 8 ml. of the mobile phase.⁷⁴ Apply 0.2 ml. of the sample to a spot 4 inches from each edge of 22.5 \times 18.25-inch Whatman No. 52 paper and let dry. Run overnight or for 16 hours, with the solvent front to within approximately 4 inches of the bottom, and with the butanol-acetic acid-water in the long direction. Dry in a circulating air oven at 40° for 2 hours and trim top and bottom. Rotate 90° and place between two 1.5 \times 25-inch strips of 1/8-inch plate glass held together with elastic bands around each end to cover the area of amino acid spots. Spray the paper with pyrophosphate buffer at pH 9.3 before the second run. Remove from the glass and dry at 40° for 30-60 minutes. Place solvent 2 partly in the tray at the bottom of the cabinet and partly in a trough at the top. Run the chromatograph overnight or for 16 hours, the solvent proceeding to within 2-3 inches of the lower edge. Dry at 40° for at least 2 hours. Prepare a spray containing 4% of xylose and 1.5% of sodium bisulfite in 0.05 *M* phosphate buffer at pH 6.5-7. Spray the paper, dry, and heat in an oven at 80°-90° for 1.5-2 hours.

Observe the spots under an ultraviolet light. Outline, cut out, and read, using exciting light at 365 m μ and transmitted light above 400 m μ .

*By diazotized sulfanilic acid.*⁷⁵ Add 1 ml. of 1% anhydrous sulfanilic acid solution in 1:9 hydrochloric acid and 1 ml. of 5% sodium nitrite solution to 10 ml. of the sample solution of amino acids. After 30 minutes,

⁷² Kazuo Satake, Tsunco Okuyama, Mochihiko Ohashi and Tomotaka Shinoda, *J. Biochem. (Tokyo)* **47**, 654-60 (1960).

⁷³ V. G. Shore and Arthur B. Pardee, *Anal. Chem.* **28**, 1479-81 (1956).

⁷⁴ A. L. Levy and David Chung, *ibid.* **25**, 396-9 (1953).

⁷⁵ Dallas Fraser and H. G. Higgins, *Nature* **172**, 459-60 (1953).

add 3 ml. of 20% sodium carbonate solution and dilute to 25 ml. with water. Read at 363 $m\mu$ for glycine and lysine and 356 $m\mu$ for arginine.

*As dioxohydrindylindonehydrindamino cadmium salts.*⁷⁶ Cut out the spots of amino acids obtained by paper chromatography and treatment with ninhydrin. To each spot, add 0.5 ml. of 0.1% cadmium chloride solution in 60% methanol. Dilute the red complex to 5 ml. with 60% methanol and read after 10 minutes.

By ascorbic acid in dimethylformamide. As reagent, dissolve 0.05 gram of ascorbic acid in 0.5 ml. of water and dilute to 50 ml. with dimethylformamide. To 0.25 ml. of aqueous sample containing 0.01-0.1 mg. of amino acid, add 1 ml. of reagent and dilute to 5 ml. with dimethylformamide. Heat at 100° for 10 minutes and cool to 15-20°. Read at 390 $m\mu$ or 525 $m\mu$. The lower wave length is more sensitive.

CARBAMYLAMINO ACIDS

Carbamylamino acids condense with the diacetylmonoxime reagent in the presence of an aromatic amine.⁷⁷ The addition of persulfate increases the sensitivity. Any substance with a ureide grouping will give some color.

The following compounds in concentration of 1 micromole form a color with the reagent: thiocarbamylglutamic acid, phenylcarbamylglutamic acid, carbamyl- α -methylglutamic acid, carbamylglutamic acid, glutamine, carbamylaspartic acid, carbamylglycine, carbamylalanine, carbamylleucine, carbamylarginine, arginine, citrulline, tryptophan, hydantoin, hydantoin-5-acetic acid, hydantoin-5-propionic acid, uric acid, uracil, adenine, urea, phenylurea, methylurea, glycocyamine, semicarbazide, and α -ketoglutaric acid semicarbazone.

The following give no color at the 20 micromole level: histidine, tyrosine, glutamic acid, methionine, ornithine, proline, asparagine, N-formylglutamic acid, N-formylaspartic acid, N-formylglycine, N-formylleucine, N-butyrylglutamic acid, N-valerylglutamic acid, carbamylproline, alanine, cystine, cysteine, N-acetylglutamic acid, N-propionylglutamic acid, glycylglycine, glycylglutamic acid, thiourea, and urethan.

Histidine and ornithine increase the color value of carbamylglutamic acid and 5 micromole of either amino acid increases the color value of 1

⁷⁶ Michael Mortreuil and Yvonne Khouvine, *Bull. soc. chim. biol.* **36**, 425-8 (1954).

⁷⁷ Seymour B. Koritz and Philip P. Cohen, *J. Biol. Chem.* **209**, 145-50 (1954).

micromole of carbamylglutamic acid by 23%. Cysteine, cystine, and methionine inhibit color formation. Uric acid increases the chromogenic equivalent upon standing. Adenosinetriphosphate does not interfere. Beer's law is followed up to 2.5 micromoles of carbamylglutaric acid. Carbamylaspartic acid in the presence of ornithine and arginine is determined by this reagent⁷⁸ (Vol. IV, pp. 153-5).

Procedure—To a 3-ml. sample, add 6 ml. of 1:1 sulfuric acid, 0.1 ml. of 1% sodium diphenylamine-*p*-sulfonate solution, and 0.25 ml. of 3% diacetyl monoxime solution. Mix, stopper with a rubber stopper containing a short section of capillary tubing, and heat at 100° for 10 minutes. Cool in running water and add 0.25 ml. of 1% potassium persulfate solution. Mix, stopper, and heat at 100° for 1 minute. Cool rapidly and protect from direct sunlight. Read at 550 $m\mu$ after 10 minutes.

PEPTIDES

Biuret is a reagent for developing peptides in urine samples,⁷⁹ and is therefore closely related to proteins (Chapter 4). Creatinine in concentration of 250 mg. per 100 ml. yields a reading of 0.005. Up to 280 mg. of uric acid per 100 ml. or 500 mg. of ammonia in the form of ammonium chloride do not interfere. Glycine in concentrations of 100 mg. per 100 ml. increases the absorbance by 0.005. Hippuric acid may be present up to 280 mg. per 100 ml. In the presence of 3 grams of glucose, an orange precipitate may appear, or the reagent may turn blue. Oxalic acid in concentrations of 100 mg. per 100 ml. decreases the absorbance by 0.01. Acetic acid inhibits color development.

Sample—Urine. To prepare the biuret reagent, add the following to 250 ml. of water, dissolving each component before the addition of the next: 19 grams of sodium potassium tartrate, 20 grams of sodium hydroxide, 5.5 grams of copper sulfate pentahydrate, 1 gram of potassium iodide. Dilute to 1 liter with water and mix. Store in an alkali resistant bottle. The reagent is stable until a black or reddish precipitate appears.

Cover the bottom of a collection vessel completely with toluene. Collect a 24 hour urine specimen and measure. To 20 ml., add 2 grams of Permutit and shake intermittently for 5 minutes. Centrifuge, and add

⁷⁸ I. Reifer and K. Toczko, *Bull. acad. polon. sci., Ser. sci. biol.* 7, 131-3 (1959).

⁷⁹ Bernard Baulikov, Eloy R. Lozano and Robert A. Castello, *Clin. Chem.* 4, 409-19 (1958).

5 ml. of water to 3 ml. of the supernatant liquid. Prepare a blank with 8 ml. of water. Add 1 ml. of 10% sodium tungstate solution and mix. Shake vigorously with 1 ml. of 1:24 sulfuric acid. Let stand for 5 minutes and centrifuge if necessary. To 5 ml. of the clear solution, add 5 ml. of biuret reagent and mix. After exactly 25 minutes, centrifuge for 5 minutes at 2500 rpm. Read at 405 $m\mu$ against the blank. If the absorbance is less than 0.05 optical density units, use 8 ml. in place of 3 ml. of urine in the procedure. If the absorbance is greater than 0.3, use less than a 3 ml. sample. To convert the absorbance from the standard curve to grams of peptide as glutathione:

$$\text{gm. per 100 ml. of diluted urine} \times \frac{8}{3} \times \frac{(\text{urine volume in ml.})}{100} =$$

gram of peptide as glutathione per 24 hours

DIPEPTIDES

When incubated with cobaltous ions in the presence of oxygen, the dipeptide forms a stable pink color, which is an oxygen-containing cobaltous compound closely related to proteins (Chapter 4). Hydrogen peroxide is the oxygenating agent and a suspension of cobaltous phosphate in borate buffer is the source of cobaltous ions.⁸⁰ Glycylglycine has an absorption peak at 520 $m\mu$ with a smaller peak at 390 $m\mu$. Alanylglycine, glycylalanine, alanylalanine and leucylglycine have absorption spectra similar to glycylglycine. Sarcosylglycine has a maximum absorption at 530 $m\mu$. Diglycylglycine produces an orange color with a maximum absorbance at 487 $m\mu$. Glycyl-L-tyrosine gives an orange yellow with a maximum at 505 $m\mu$, and sarcosylglycine, a blue with a maximum at 530 $m\mu$. Glycinamide and glycylproline do not form colors under these conditions.

The procedure is not applicable to samples containing cystine, tyrosine, tryptophan, or histidine, since these amino acids give a color with the reagent. Most other amino acids do not interfere. Over 200 micromoles of phosphate depresses the color slightly.

Procedure—Macro. To prepare the suspension of cobaltous phosphate in borate buffer at pH 9.1, add 3 volumes of 2.6% cobaltous chloride solution to 2 volumes of 3% trisodium phosphate solution. Mix thoroughly

⁸⁰ E. M. Crook and B. R. Rabin, *Biochem. J.* **68**, 177-82 (1958).

and dilute to 10 volumes with 3.8% borax solution. Shake well before using.

To a sample of up to 10 ml. containing 80 micromoles of dipeptide or hydrolysis products, add 3 drops of 0.1% ethanolic thymolphthalein solution. Add 2% sodium hydroxide solution until the solution turns blue. Add the cobaltous phosphate suspension and heat at 100°. At the end of each of three 10-minute intervals, slowly add 0.5 ml. of 0.3% hydrogen peroxide with constant shaking. Remove the solution from the bath during addition of the hydrogen peroxide. After the final addition of peroxide continue heating for 30 minutes. Cool to room temperature and dilute to 25 ml. with water. Filter and read with an Ilford 624 green filter.

Micro. The sample should contain 20 micromoles of dipeptide or its hydrolysis product. Add 1 drop of 0.1% ethanolic thymolphthalein solution. Add 0.4% sodium hydroxide solution until the solution is blue. Add 2 ml. of the cobaltous phosphate suspension and follow the macro procedure using three 0.25-ml. portions of 0.15% hydrogen peroxide. Dilute to 5 ml. and centrifuge before reading.

AMINO ACID HYDROXAMIDES

Amino acid hydroxamides are determined by ferric chloride.⁸¹ Beer's law is followed for 0.2-1 micromole for leucine, alanine, glycine, tyrosine, tryptophan, and methionine hydroxamides. The final pH of the solution should be 0.7-0.9.

Procedure—To obtain trichloroacetic acid at pH 0.9, neutralize the saturated solution with sodium hydroxide. In the cold, this keeps for 1 week. The 3-ml. sample solution should be at pH 7 and contain 3.3% of hydroxylamine. Add 1.4 ml. of the trichloroacetic acid at pH 0.9 and 0.6 ml. of 32% ferric chloride solution. Centrifuge the protein precipitate and read after 5-20 minutes at 520 m μ .

DIAMINO ACIDS

Straight-chain amino acids containing two amino acid groups produce a yellow color with ninhydrin.⁸² By this method, the acid can be meas-

⁸¹ Richard S. Schweet, *Biochim. et Biophys. Acta* **18**, 566 (1955).

⁸² Elizabeth Work, *Biochemical J.* **67**, 416-23 (1957).

ured directly in enzyme reaction mixtures and in acid eluates from ion-exchange columns. Tryptophan, cystine, ornithine, proline and lysine must not be present in large concentrations. Beer's law is followed for 0.006-0.115 mg. at 345, 340, and 440 $m\mu$.

Procedure—Dissolve 250 mg. of ninhydrin in 4 ml. of 58% phosphoric acid. Mix a 0.5-ml. sample containing up to 3 micromoles of the diamino acid with 0.5 ml. of glacial acetic acid and 0.5 ml. of ninhydrin reagent. Cover, and heat at 100° for 5 minutes. Cool rapidly to room temperature. Add 3.5 ml. of glacial acetic acid. Read at an appropriate wave length from Table 16 against a reagent blank.

TABLE 16. ABSORPTION MAXIMA FOR
DIAMINO ACIDS WITH NINHYDRIN

<i>Compound</i>	<i>Absorption maximum</i>
Diaminosebacic acid	330-70 $m\mu$
Diaminoazelaic acid	330 $m\mu$
Diaminosuberic acid	325 $m\mu$
Diaminopimelic acid	345 $m\mu$
Lysine	340 $m\mu$
Diaminoadipic acid	515 $m\mu$
Ornithine	515 $m\mu$
Diaminoglutaric acid	325 $m\mu$
Diaminobutyric acid	330 $m\mu$
Diaminosuccinic acid	330 $m\mu$
Homocystine	325 $m\mu$
Djenkolic acid	360 $m\mu$
Cystine	345 $m\mu$
Cystinamine	340 $m\mu$
Lanthionine	360 $m\mu$
Cystathionine	340 $m\mu$
s-(β -Aminoethyl)cysteine	340 $m\mu$

GLYCINE, AMINOACETIC ACID

Glycine is determined by the color reaction with pyridine and *p*-nitrobenzoyl chloride.⁸³ Glycine is also developed by electrophoresis in aqueous acetic-formic acid. The spots are developed with ninhydrin in alkaline ethanol solution after drying at 80°. The relative mobility dis-

⁸³ C. J. Umberger and F. F. Fiorese, *Clin. Chem.* **9**, 79-90 (1963).

tinguishes the glycine. This method will detect 2.5 γ in 2.5 ml. of sample. The spot is eluted with cadmium chloride in aqueous methanol and read at 505 m μ .⁸⁴

Glycine by chromotropic acid (Vol. IV, pp. 112-3) is read at 550 m μ .⁸⁵ For determination of glycine as formaldehyde in the presence of alanine, see page 251. As a specific reaction, it can be converted to glycollic acid with nitrous acid. After removal of excess acid, it is converted to formaldehyde and determined by chromotropic acid.⁸⁶ A specific reaction is that with 1,2-naphthoquinone-4-sulfonate.⁸⁷ Beer's law is followed at 0.1-0.6 mg. of glycine.

Glycine gives a red color with pyridine and ethyl chloroformate.⁸⁸ Other amino acids need not be separated. Thus the absorbance is increased by only 0.07 by an equal amount of DL- α -alanine, β -alanine, L(+) glutamic acid, L(-) leucine, DL-isoleucine, DL-lysine monohydrochloride, DL- β -phenylalanine, and DL-threonine. The drying temperature and reaction immediately thereafter must be observed in order to avoid low results, because the color is unstable in the presence of moisture. Glycine anhydride produced by overheating does not react. Excess pyridine gives a yellow color but the excess is destroyed by excess ethyl chloroformate. Fading is about 0.001 unit per minute. Readings can be as low as 3 microgram per ml. of sample. The method is applicable to acid hydrolysates of gelatine and collagen.

Procedure—By *p*-nitrobenzoyl chloride. Dilute 0.1 ml. of sample containing no more than 0.0125 mg. of glycine to 2.5 ml. with pyridine. Prepare a water blank. Add 0.4 each to cuvetts containing exactly 115.2 mg. of *p*-nitrobenzoyl chloride. Agitate to dissolve. After 2 minutes, dilute each to 5 ml. with chloroform. Read the sample against the blank at 480 m μ .

By *chromotropic acid*. Mix a 5-ml sample containing about 0.5 mg. of glycine with 2 ml. of fresh 7% sodium nitrite solution. Add 2 ml. of 1:35 sulfuric acid and mix. Heat at 100° for 45 minutes. Cool, and dilute to 10 ml. with water. Add 0.2 ml. of this solution to 0.02 gram of solid

⁸⁴ J. Saint-Blancard and J. Storek, *J. ann. pharm. Franc.* **18**, 711-14 (1960).

⁸⁵ E. Orlando and L. Ferrari, *Giorn. biochim.* **3**, 147-54 (1954).

⁸⁶ J. Giroux and A. Puech, *Ann. Pharm. Franç.* **21**, 469-76 (1963).

⁸⁷ J. P. S. Aarin, R. B. Chakravarty, G. K. Ray and B. Kukerji, *Indian J. Pharm.* **25**, 375-6 (1963).

⁸⁸ R. L. Sublett and J. P. Jewell, *Anal. Chem.* **32**, 1841-2 (1960); J. P. Jewell, Mary J. Morris and R. L. Sublett, *ibid.* **37**, 1034-5 (1965).

stannous chloride. Add 5 ml. of concentrated sulfuric acid, then 0.1 ml. of 5% aqueous chromotropic acid. Shake. At this stage the solution should be no darker than pale amber. Heat at 100° for 20 minutes and store in the dark for 45 minutes. Read at 580 m μ .

By 1,2-naphthoquinone. Elixir. Dilute 1 ml. of aminoacetic acid elixir to 100 ml. Dilute 3 ml. of this to 50 ml. To 3 ml. of this dilution, add 1 ml. of 8% sodium hydroxide solution. Add 1 ml. of fresh 1% solution of 1,2-naphthoquinone-4-sulfonate. Develop at 80° for 20 minutes. Cool and dilute to 8 ml. Add 1 ml. of acetone, 1 ml. of 1:3 hydrochloric acid, and 0.5 ml. of 0.1 N sodium thiosulfate solution. Dilute to 20 ml. with acetone and read at 540 m μ .

By ethylchloroformate and pyridine. Take a sample containing 0.003-0.015 mg. of glycine in a 15-ml. graduated centrifuge tube. Dry at 125-130° for 2-3 hours. Immediately mix with 0.1 ml. of pyridine at 100° by stirring. Add 0.2 ml., 0.2 ml., and 0.1 ml. of ethylchloroformate. In each case, add dropwise and stir after each addition. During this development, maintain the temperature at 100°. Continue to heat until the maximum red color is obtained, usually 1 minute. Immediately separate the sample from the ethylchloroformate layer by aspiration to avoid development of a green color. Dilute the sample to 10 ml. with butanol, cool, and read within 5 minutes at 420 m μ against butanol.

HIPPURIC ACID, BENZOYLGLYCINE, BENZOYLAMINOACETIC ACID

Hippuric acid (see Vol. IV, p. 114) is separated from urine by passage through a cation-exchange resin, Dowex 50-X8 in the hydrogen form. Creatinine and amino acids are retained on the resin. Uric acid is eluted along with hippuric acid. Hippuric acid is determined in the presence of uric acid by additive absorbaney.⁸⁹ Beer's law is followed for hippuric acid at 232 m μ for 0.001-0.025 mg. per ml. Benzoic acid interferes due to an absorption maximum at 224 m μ . By reading at 246 m μ where hippuric and benzoic acid have their maximum difference, and at 220 m μ where they have their minimum difference, each can be determined in the presence of the other.⁹⁰

Hippuric acid is converted to a deep orange azolactone, α -phenyl-4-(*p*-dimethylamino)benzal-5-oxazolone, by treatment with acetic anhy-

⁸⁹ Howard C. Elliott, Jr., *Anal. Chem.* **29**, 1712-15 (1957).

⁹⁰ H. P. Rieder, *Clin. Chim. Acta* **2**, 497-501 (1957).

dride and *p*-dimethylaminobenzaldehyde.⁹¹ The reagents are sprayed on a paper chromatogram of the sample and the orange azolactone is eluted with methanol. In concentrated urine samples, yellow spots developed on standing. Beer's law is followed at 460 $m\mu$ for 0.01-0.1 mg. of hippuric acid. Amino acids, taurine, *p*-aminobenzoic acid, niacin, and isonicotinic hydrazide do not interfere. The reagent also reacts with benzoyl- β -alanine and benzoyltaurine which therefore can interfere.⁹² Hippuric acid in 70% sulfuric acid fluoresces with a sensitivity to 0.002 mg. per ml.⁹³

Procedure—Urine in the ultraviolet. To prepare the cation-exchange resin, stir 50-100 mesh Dowex 50-X8 in 1:10 hydrochloric acid followed by 4% sodium hydroxide solution alternately for 1 hour each, three times. Wash with water and decant until the water is chloride-free as tested with 1% silver nitrate solution. Pour 20 ml. of the moist resin into a chromatographic column 12 mm. in diameter and allow water to flow over the column until the pH of the water is 7. Determine the recovery of a known sample of hippuric acid from the resin.

If the recovery is not 100%, stir 50 ml. of the resin for 1 hour with 100 ml. of 0.1% hippuric acid solution. Pour the resin into the column and wash free of excess acid with water. This procedure usually requires 200 ml. of water.

Add 1 ml. of urine sample to the column followed by 5 ml. of water, added slowly. After 5 minutes, elute hippuric acid and uric acid with water. Collect 200 ml. Stopper, and mix by inversion. Read at 232 and 287 $m\mu$ against water. Convert the 287 $m\mu$ reading due to uric acid to the corresponding value at 232 $m\mu$ and subtract from the total 232 $m\mu$ reading. For 232 $m\mu$, curve $Y = 0.00186 + 49.545 X$; and for 287 $m\mu$, curve $Y = 0.00038 + 65.63 X$; in which Y = absorbance and X = concentration.

Urine as an azolactone. Prepare a paper chromatogram of the sample on 1-inch strips of Whatman No. 1 filter paper, using a 4:1:1 butanol-acetic acid-water solvent system. The R_F value for hippuric acid is 0.79. Dry the strips. Spray with 4% *p*-dimethylaminobenzaldehyde solution in acetic anhydride containing a few crystals of sodium acetate. Heat in an

⁹¹ George W. Gaffney, Kurt Schreier, Nicola Di Ferrante and Kurt I. Altman, *J. Biol. Chem.* **206**, 695-8 (1954).

⁹² Kenji Imanishi, *Nagasaki Igakkai Zasshi*, **29**, 809-12 (1954).

⁹³ See G. L. Ellman, A. Burkhalter and J. La Don, *J. Lab. Clin. Med.* **57**, 813-18 (1961).

oven at 130-150° for 1-2 minutes. Cut out the spots containing 0.01-0.1 mg. of hippuric acid and extract with three 2-ml. portions of methanol. Read immediately at 460 m μ .

In urine samples containing 0.01-0.1 mg. of hippuric acid, the sample may be chromatographed directly. For lower concentrations, extract the urine by continuous extraction with ethyl acetate for 24 hours. Evaporate the extract and take up the residue in ethanol. Chromatograph the ethanol solution.

SERINE, 2-HYDROXY-1-AMINOPROPIONIC ACID

The formaldehyde formed by oxidation of serine is developed with chromotropic acid (see Vol. IV, p. 106, 117-9). Bisulfite is substituted for arsenite used in other procedures to reduce excess periodate, as the results are reproducible.⁹⁴ Beer's law is followed for 4×10^{-2} micromoles of serine per ml.

The following compounds give a red color when treated with periodate and chromotropic acid: dimethylaminoethanol, methylaminoethanol, ethanolamine, glyoxal, and threonine. Methionine, betaine, choline, creatine, creatinine, homocysteine, homocystine, guanidoacetic acid, ethanol, methanol, formic acid, diphosphopyridine nucleotide, cytochrome-C, flavin-adenine dinucleotide, cysteine, cystine, urea, and monomethylamine do not interfere.

Mixtures of serine and threonine react with periodate in neutral solution to give formaldehyde and acetaldehyde, respectively. Similarly, glycine and alanine so react with ninhydrin. In either case, the aldehydes can be separated and determined with chromotropic acid at 570 m μ (Vol. III, p. 259) or with *p*-hydroxybiphenyl at 560 m μ (Vol. III, pp. 262-3).

Procedure—As formaldehyde by chromotropic acid. Washed mitochondrial suspension and other tissue particulate preparations. To prepare the chromotropic acid reagent, dissolve 0.5 gram of 1,8-dihydroxynaphthalene-3,6-disulfonate in 50 ml. of water and 200 ml. of 1:98 sulfuric acid.

Treat the sample with an equal volume of 20% trichloroacetic acid

⁹⁴ Wilhelm R. Frisell, Lloyd A. Meech and Cosmo G. Mackenzie, *J. Biol. Chem.* 207, 709-16 (1954); Makoto Hayashi, Komei Miyaki, and Tsutomu Unemoto, *Chem. Pharm. Bull. (Tokyo)* 8, 904-7 (1960); L. W. Wheeldon, M. Brinley and D. A. Turner, *Anal. Biochem.* 4, 433-43 (1962); Makoto Hayashi, Yoshinori Nakajima, Keizo Iizawa and Komei Miyaki, *Chem. Pharm. Bull., Japan* 11, 1200-2 (1963).

solution and centrifuge. Dilute an aliquot of the supernatant liquid containing 2-6 micromoles of serine to 3 ml. with water. Add 1 drop of a half-saturated methyl red solution in 1:239 hydrochloric acid. Pipet 20% sodium hydroxide solution into the sample until the solution is just basic to the indicator. Add 1 ml. of 1.5% sodium metaperiodate solution. After 5 minutes, add 10% trichloroacetic acid solution dropwise to the exact end-point of the indicator.

Add 1 ml. of 10% sodium bisulfite solution and dilute to 25 ml. with water. Transfer an aliquot equivalent to at least 0.1 micromole of the original serine and dilute, if necessary, to 1 ml. with water. Develop the formaldehyde color by heating with 10 ml. of chromotropic acid reagent at 100° for 30 minutes. Cool to room temperature and add 1 ml. of a half-saturated thiourea-water solution to reduce the effect of any red color contributed by the reagents. Read at 570 $m\mu$.

By dinitrofluorobenzene. See ethanolamine, page 103.

CYSTEINE, β -MERCAPTOALANINE, 2-AMINO-3-MERCAPTOPROPIONIC ACID

In the reaction of fluoropyruvate and thiol compounds, the —SH groups disappear and equivalent amounts of hydrofluoric acid are liberated. Cysteine and related compounds give reaction products with an absorption peak at 300 $m\mu$.⁹⁵ The stability of the cysteinefluoropyruvic acid compounds decreases with alkalinity and the peak of the absorption spectrum is displaced to 270 $m\mu$ with increasing acidity.

There is no interference from mercaptans without unsubstituted amino groups, other amino acids except homocysteine, or high salt concentrations. Reducing agents interfere. An excess of fluoropyruvate is required to bind all thiol compound present. Beer's law is followed up to 16 micromoles $\times 10^2$ per ml. As low as 0.02 micromole per ml. can be determined.

An equimolecular reaction of cysteine and noradrenochrome changes the pink color of the reagent to yellow.⁹⁶ No other mercapto compound is known to interfere. Cysteine is also determined by its reaction with decolorized thiofluorescein.⁹⁷

Cysteine forms a complex with lead ion absorbing at 262 $m\mu$ in acetate

⁹⁵ Y. Avi-Dor and J. Mager, *J. Biol. Chem.* 222, 249-58 (1956); Y. Avi-Dor and Rebecca Lipkin, *ibid.* 233, 69-72 (1958).

⁹⁶ Sidney Roston, *Anal. Biochem.* 6, 486-90 (1963).

⁹⁷ P. Dubouloz, J. Fondarai and R. Pavone-Marville, *Anal. Chim. Acta* 26, 249-52 (1962).

buffer for pH 5.0-5.5.⁹⁸ Up to 2 millimoles of lysine, leucine, glutamic acid, and threonine do not interfere. More than 10 millimoles of phenylalanine, tyrosine, and tryptophan do not interfere. Cysteine can be determined as cysteic acid, page 271.

Procedure—*By fluoropyruvic acid.* To 0.2 ml. of 0.2% fluoropyruvic acid solution, add 0.1-1 ml. of a sample solution containing around 0.1 mg. of cysteine per ml. Dilute to 2.5 ml. with water. Prepare 2 control cells. Cell 2 contains 0.2 ml. of fluoropyruvic acid solution diluted to 2.5 ml. with water and cell 3 contains 2.5 ml. of water. Add 0.5 ml. of 0.5 *M* tris buffer at pH 8 to each cell. The reading of the sample cell against cell 2 at 300 $m\mu$ should be close to zero. Read the sample after 5 minutes at 300 $m\mu$.

By thiofluorescein. Dilute a 0.2% solution of thiofluorescein in ethyl acetate buffered at pH 8 with disodium phosphate with 4 volumes of water. Decolorize by exposure to light for 15 minutes. This is appropriately by a 100-watt bulb. Prepare a buffer by adding 1.49 grams of potassium chloride to 100 ml. of 1.96% phosphoric acid. Mix 1 ml. of sample solution containing 0.0012-0.24 mg. of cysteine with 1.2 ml. of the buffer, 1.2 ml. of 7.6% solution of sodium tetraborate decahydrate, and 5 ml. of the decolorized reagent. Incubate at 50° for 30 minutes and cool to 25°. After 15 minutes, read at 580, 585, and 590 $m\mu$.

By noradrenochrome. As reagent, mix 0.1 ml. of 0.25% potassium ferricyanide solution, 0.2 ml. of 0.01% solution of noradrenaline in 1:1000 hydrochloric acid, 1 ml. of 0.1 *M* phosphate buffer for pH 7.5, and 3.15 ml. of water. Discharge the yellow color of excess ferricyanide after 2 minutes by dropwise addition of 0.1 ml. of 0.17% ascorbic acid solution.

Mix the cysteine sample with the noradrenochrome reagent. After 10 minutes, add 0.15 ml. of 0.02% solution of sodium bisulfite. Read at 414 $m\mu$ within 0.5-1.0 minute.

CYSTINE, DICYSTEINE, 3,3'-DITHIObis(2-AMINO PROPIONIC ACID)

For determination by decolorized thiofluorescein, cystine is reduced to cysteine with potassium cyanide.⁹⁹ For determination in protein, the

⁹⁸ Jun Mizuguchi, Fujio Takahashi and Yoshiaki Saino, *J. Chem. Soc. Japan, Pure Chem. Sect.* **83**, 957-8 (1962).

⁹⁹ P. Dubouloz, J. Fondarai and R. Pavone-Marville, *Anal. Chim. Acta* **26**, 249-52 (1962).

sample is oxidized by peroxyformic acid at -10° .¹⁰⁰ The oxidized protein is hydrolyzed with 1:1 hydrochloric acid to liberate cysteic acid. This is separated by chromatographing on Dowex 2-X10 and elution with chloroacetic acid solution. Thereafter, it is determined by ninhydrin. Cystine can be determined as cysteic acid, page 271.

Procedure—Most of the required reagents are described under cysteine. Mix 1 ml. of sample containing 0.0024-0.48 mg. of cystine, 1.2 ml. of the buffer, 0.8 ml. of the borate solution, and 0.8 ml. of a 1.3% solution of potassium cyanide in the buffer. Complete as for cysteine, from "Incubate at 50° . . ."

PHENYLALANINE, 2-PHENYL-1-AMINOPROPIONIC ACID

For phenylalanine, proteins are precipitated with picric acid. If trichloroacetic acid is used as the precipitant, it is difficult to remove from the filtrate. The amino acids are isolated from serum samples by an ion-exchange resin and are eluted with ammonium hydroxide. Beer's law is followed at $555\text{ m}\mu$ up to 67 mg. per 100 ml. of sample.

L-Phenylalanine is oxidized to phenylpyruvic acid by L-amino oxidase from snake venom.¹⁰¹ In the presence of arsenate and borate ions, the α -keto acid is converted to an enol-borate complex, which has high absorption in the ultraviolet. Catalase protects the keto acid from peroxide formed in the oxidative deamination by L-amino oxidase. Histidine does not react under the conditions of the procedure. Tryptophan and tyrosine can also be measured by this procedure.

By use of a larger serum sample, the sample is deproteinized with perchloric acid precipitation, followed by neutralization, and removal of perchlorate. Without the deproteinization, turbidity occurs. For elevated serum levels of phenylalanine, the method is rapid and specific, while in normal serum, there is greater interference from tryptophan and tyrosine. Beer's law is followed at $308\text{ m}\mu$ for 0.002-0.03 mg. of phenylalanine.

¹⁰⁰ C. deBelsunce and R. Pion, *Ann. Biol. Biochem. Biophys.* **3**, 191-9 (1963); cf. E. Schram, S. Moore, and E. J. Bigwood, *Biochem. J.* **57**, 33-7 (1954); S. Moore, *J. Biol. Chem.* **238**, 235-7 (1963); cf. R. J. Treacher, *Biochem. J.* **90**, 494-8 (1964).

¹⁰¹ Bert N. La Du and Patricia J. Michael, *J. Lab. Clin. Med.* **55**, 491-6 (1960); Bert N. La Du, R. Rodney Howell, J. Michael Patricia and Eva K. Sober, *Pediatrics* **31**, 39-46 (1963); A. Knapp, M. Hothmann, B. Richter and W. Foerster, *Deut. Gesundheitsw.* **18**, 2285-9 (1963).

Phenylalanine is nitrated and the nitrated product is reduced with hydroxylamine and ammonium hydroxide.¹⁰² (Vol. IV, pp. 120-2).

When ninhydrin-treated paper chromatograms are treated with dilute sodium bicarbonate solution, a blue color forms. The ninhydrin colors of the other amino acids are washed away with water. Phenylalanine is then extracted with 1-butanol and read at 600 m μ .¹⁰³ Beer's law is followed for 0.01-0.05 mg. of phenylalanine.

Phenylalanine is developed fluorimetrically in a buffer for 5.8 when treated with ninhydrin, L-leucyl-L-alanine, and a modified Fehling solution.¹⁰⁴ Accuracy is to $\pm 5\%$. Other dipeptides are also effective in intensifying the fluorescence of phenylalanine and ninhydrin.

Procedure—*By catalase and venom serum.* To prepare the snake venom L-amino acid oxidase, suspend the dried venom in water so that the concentration is 10 mg. per ml. Centrifuge, and use the supernatant liquid. Store at 0-5°. To determine the activity of the L-amino acid oxidase preparation, test with 0.1 ml. of phenylalanine standard containing 0.0165 mg. of phenylalanine, following the procedure.

Dilute crystalline beef liver catalase 1:5 with 0.2 M phosphate buffer for pH 6.5. Store at 0-5°. To prepare the arsenate-phosphate buffer, prepare a saturated sodium arsenate solution in 0.2 M phosphate buffer and adjust the final pH to 6.5 with 1:1 hydrochloric acid. Dissolve 61.8 grams of boric acid in 624 grams of saturated sodium arsenate solution. Adjust the pH to 6.5 with hydrochloric acid and dilute to 1 liter.

Allow the sample to clot. Heparinized samples develop turbidity. To three 1.2-ml. cuvetts, add the following:

	C ₁	E ₁	E ₂
Phosphate, buffer, 0.2 M, pH 6.5	0.39	0.39	0.39
Arsenate-phosphate, pH 6.5	0.5	—	—
1 M borate in arsenate	—	0.5	0.5
Catalase, 1:5 dilution	0.01	0.01	0.01
Venom, 10 mg. per milliliter (serum added later)	0.10	0.10	0.10

Mix, and read E₁ and E₂ against C₁ at 308 m μ . This reading should be approximately zero. Add 0.1 ml. of serum to each cuvet and read at

¹⁰² Richard J. Henry, Charles Sobel and Neil Chiamori, *A.M.A.J. Diseases Children* **94**, 604-8 (1957).

¹⁰³ Arthur E. Pasicka and Joseph F. Morgan, *Anal. Chem.* **28**, 1964-6 (1956).

¹⁰⁴ M. W. McCaman and E. Robins, *J. Lab. Clin. Med.* **59**, 885-90 (1962).

308 $m\mu$ at 1-2 minute intervals for 10 minutes. When there is no further change in the optical density, reading the enzyme reaction is complete. Read at 308, 330, and 350 $m\mu$. Readings are taken at three wave lengths to correct for absorption of keto acids from tyrosine and tryptophan, since these acids are also oxidized by oxidase. Calculate the micrograms of phenylalanine per ml. of serum, based on a sample of:

$$0.1 \text{ ml. of serum} = \frac{Y}{0.031} \times 10$$

in which Y equals $1.23A - 2.26B + 2.15C$, where A is the reading at 308 $m\mu$; B is the reading at 330 $m\mu$; and C is the reading at 350 $m\mu$.

By hydroxylamine hydrochloride after nitration. Serum or plasma. To 10 ml. of 1% picric acid solution, slowly add, with constant mixing, 2 ml. of serum or plasma. After 5 minutes, centrifuge. Suspend Dowex 50-X8 resin in water and pour into a column 10 mm. in internal diameter containing a glass wool plug at the bottom. Pack the column by gravity to a depth of 5 cm. and place a glass wool plug at the top.

Acidify 9 ml. of the protein-free supernatant liquid to pH 1-2 with 1:10 hydrochloric acid and pass the sample through the resin. Do not allow the level of the liquid to fall below the upper surface of the upper glass wool plug. Wash the column with 10 ml. of 1:1100 hydrochloric acid followed by eight 5-ml. portions of water or until the eluate is colorless and free of picric acid. The column may be washed by suction.

Elute the amino acids with 15 ml. of 1:9 ammonium hydroxide. Discard the first 3 ml. of eluate and collect 12 ml. Evaporate the eluate to 2 ml. at 100° and finally evaporate to dryness at 100° under nitrogen or dry compressed air.

Dry 1 ml. of standard phenylalanine solution containing 0.36 mg. at 100° under nitrogen or compressed air. To the sample, standard, and an empty tube, add 0.4 ml. of 20% potassium nitrate solution in concentrated sulfuric acid and heat at 100° for 20 minutes. Cool in ice water, add 2.5 ml. of water, and cool in ice water. Add 0.5 ml. of 30% hydroxylamine hydrochloride solution, mix, and let stand in ice water for 5 minutes. Dilute with cold concentrated ammonium hydroxide to 5 ml., mix, and read the sample and standard against the reagent blank at 555 $m\mu$.

By ninhydrin. As a solvent for paper chromatography, add 30 ml. of glacial acetic acid to 250 ml. of 1:1 *n*-butanol-water. Shake. Use the

bottom layer of acetic acid saturated with butanol at the bottom of the tank and the top butanol layer in the trough for running the chromatogram.¹⁰⁵ Evaporate a 5-ml. sample to dryness *in vacuo* over concentrated sulfuric acid and dissolve in 0.2 ml. of water.

Using a 0.01-ml. aliquot, develop a one-dimensional paper chromatogram by the descending method at room temperature for 18 hours. Dry at 110° for 2-3 minutes and redevelop in the same solvent for 18 hours in the same dimension. Dry at 110° for 2-3 minutes and spray with 0.4% ninhydrin solution in water-saturated 1-butanol or 95% ethanol. Dry at room temperature for 5-10 minutes. Heat at 110° for 3 minutes and dip the chromatogram into 1% sodium bicarbonate solution. The phenylalanine develops as a deep blue spot.

Cut out the phenylalanine area and then cut into several strips. Shake the paper with 5 ml. of deionized water for 1 minute. Decant the liquid and repeat the washing. Drain and add 5 ml. of butanol and shake for 10 minutes. Read the extract at 600 m μ against 1-butanol.

Fluorescently. Mix equal volumes of serum or heparinized plasma with 10% trichloroacetic acid and centrifuge. Mix 0.01 ml. of the supernatant liquid with 0.1 ml. of 0.3 M succinate buffer for pH 5.8. Add 0.04 ml. of 0.5% ninhydrin solution and 0.02 ml. of 1% solution of L-leucyl-L-alanine. Incubate at 60° for 2 hours. As a copper reagent, dissolve 1.6 grams of sodium carbonate, 0.065 gram of sodium potassium tartrate, and 0.06 gram of copper sulfate pentahydrate separately in 300 ml. portions of water, mix in the order cited, and dilute to 1 liter. Add 1 ml. of this reagent and compare the fluorescence with that of a standard activated at 365 m μ and read at 520 m μ .

2-(p-HYDROXYPHENYL)-1-AMINOPROPIONIC ACID, TYROSINE

The reaction between 1-nitroso-2-naphthol and tyrosine yields a yellow product suitable for colorimetric estimation (cf. Vol. IV, pp. 122-7). When the fluorescence of the product is measured, the method shows greater sensitivity and specificity than reading in the ultraviolet.¹⁰⁶ As little as 0.001 mg. of tyrosine can be measured, and the range is up to 0.015 mg. This procedure does not distinguish between tyramine and

¹⁰⁵ A. J. Woiwod, *J. Gen. Microbiol.* **3**, 312-8 (1949).

¹⁰⁶ T. P. Wankes and Sidney Udenfriend, *J. Lab. Clin. Med.* **50**, 733-6 (1957); Michel Duval and Jean Delga, *Ann. farm. franç* **19**, 94-103 (1961).

tyrosine. A method developed for tyrosine in gelatin uses a promoter for greater reliability by direct reading with α -nitroso- β -naphthol.¹⁰⁷ Gelatin contains 0.2-0.8% of tyrosine.

Tyrosine in casein, edestin, ovalbumin, fibrin, gliadin, gelatin and in iodinated protein preparations can be determined by 1-nitro-2-naphthol in nitric acid solution by reading at 490 m μ .¹⁰⁸ Excess nitric acid, on heating with the amino acid at 100°, destroys the color. Ethanol reduces the color intensity considerably, and ethanol and other alcohols are not suitable solvents for the nitrosonaphthol reagent. Addition of 0.5 M sodium chloride stabilizes the red pigment.

Ferrous, cobalt, and nickel ions interfere by complexing the reagent. The protein-precipitating reagents, tungstic, and molybdic acids, interfere, particularly if phosphate is present. Ferric ion and cupric ion do not interfere. Trichloroacetic acid has no effect on the reaction. Strong reducing agents such as cysteine or ascorbic acid inhibit color development. To remove this interference, warm the sample to 60° with 1 drop of concentrated nitric acid before adding the reagent. Glucose may be present up to 0.02 mg. per ml. Tryptophan slightly inhibits color formation when present in large amounts. *p*-Hydroxyphenylpyruvic acid, glycytyrosine and *p*-cresol also give the red color with the reagent. The absorption spectrum shows a maximum at 320 m μ and at 500 m μ . At the latter wave length, Beer's law is followed for 0-0.08 mg. of tyrosine.

The fluorescence of tyrosine is read after acid hydrolysis of the sample. By this procedure, tyrosine is measured in the presence of tryptophan, which is destroyed by acid hydrolysis.¹⁰⁹ Without hydrolysis, interfering substances are precipitated by mercuric chloride and sodium carbonate or with silver nitrate and barium hydroxide. Thereafter, the reaction is diazotized and developed with sulfanilic acid in acid solution.¹¹⁰

Alternatively, the compound is coupled in the 3- and 5-positions with diazotized 2,4,6-tribromoaniline in a cold acid medium. On heating in boiling water, the yellow compound is changed over a period of 35 minutes to a red condensed ring. Then one reads 0.03-0.15 mg. in 5 ml. at 525 m μ .¹¹¹

A red color in alkaline solution with 1-nitroso-2-naphthol is applicable

¹⁰⁷ W. G. Corbett, A. W. Kenchington and A. G. Ward, *Biochem. J.* **84**, 468-74 (1962).

¹⁰⁸ L. K. Ramachandran and P. S. Sarma, *J. Sci. Ind. Research* **101**, 246-53 (1951); J. H. Ottaway, *Biochem. J.* **68**, 239-44 (1958).

¹⁰⁹ Daniel E. Duggan and Sidney Udenfriend, *J. Biol. Chem.* **223**, 313-19 (1956).

¹¹⁰ M. Milenkovic, *Veterinaria (Sarajavo)* **10**, 241-4 (1961).

¹¹¹ C. Dumazert, C. Ghiglione, and M. Bozzi-Tichadou, *Bull. soc. chim. biol.* **38**, 1083-8 (1956).

to 3% in the range of 0.05-0.3 mg. of tyrosine.¹¹² There is a corresponding reaction in acid solution in the presence of formaldehyde.¹¹³ In potato juice, the tryptophan is precipitated with mercuric sulfate, leaving the tyrosine in solution for determination.¹¹⁴ Tyrosine is also determined by phosphotungstic-phosphomolybdic acid, the Folin-Ciocalteu reagent, for reading at 750 m μ ¹¹⁵ (cf. Vol. IV, pp. 126-7). For tyrosine by L-amino-oxidase and catalase, see page 254.

Procedure—*Fluorescently, by 1-nitroso-2-naphthol. Plasma.* To prepare the nitric acid reagent, mix 24.5 ml. of 1:5 nitric acid with 0.5 ml. of 2.5% sodium nitrite solution. Dilute a 1-ml. heparinized sample to 4 ml. with water. Add 1 ml. of 30% trichloroacetic acid solution and centrifuge after 10 minutes. To 2 ml. of the deproteinized plasma, add 1 ml. of 0.1% 1-nitroso-2-naphthol solution in 95% ethanol, and 1 ml. of nitric acid reagent. Stopper, shake, and heat at 55° for 30 minutes. Cool, and shake with 10 ml. of ethylene dichloride to extract unchanged nitrosonaphthol reagent. Read the fluorescence of a portion of the aqueous layer at 570 m μ with activation at 460 m μ .

Protein. Heat a 20-50 mg. sample with 1 ml. of 1:4 sulfuric acid, following the procedure for basic hydrolysis under tryptophan, on page 263, starting at "Heat in an open tube . . ." and ending with "drying a separate sample *in vacuo* at 100°." Cool. Neutralize by adding 1:10 ammonium hydroxide dropwise and dilute to 25 ml. with water. Develop tyrosine by fluorescence as described for plasma above.

General. To prepare the stabilizing agent, add 75 ml. of acetone to 50 ml. of 5.8% sodium chloride solution. Dilute to 300 ml. and add 5 ml. of 8% ferric chloride hexahydrate solution immediately before use. To 2 ml. of sample containing 0.01-0.2 mg. of tyrosine, add 0.1 ml. of 0.3% 1-nitroso-2-naphthol solution in acetone. Mix and heat at 60° for 5 minutes. Remove from the bath and add 4 drops of concentrated nitric acid. Heat at 60° for 1 minute. Mix by swirling and let cool to room temperature. After 5 minutes, add 6 ml. of stabilizing agent. The final solution should now contain 0.12 *M* sodium chloride and 25% of acetone. Read within 10 to 45 minutes at 500 m μ .

¹¹² J. P. Zalta and Y. Khouvine, *ibid.* **35**, 697-701 (1953).

¹¹³ L. K. Ramachandran and P. S. Sarma, *J. Sci. Ind. Research (India)* **101**, 246-53 (1951).

¹¹⁴ Th. Breyhan, *Z. anal. Chem.* **143**, 241-4 (1954).

¹¹⁵ Susumu Teurufuji, *J. Japan. Biochem. Soc.* **36**, 277-8 (1964).

Photometrically by 1-nitroso-2-naphthol. As the promoter solution, mix 2 ml. of 0.03% 1-nitroso-2-naphthol in 0.12% sodium hydroxide solution with 1 ml. of solution containing about 0.1 mg. of tyrosine, 6 ml. of water, 1 ml. of 1:5 nitric acid, and 3 ml. of concentrated hydrochloric acid. Heat at 70° for 3 minutes. Add 50 ml. of 1:5 nitric acid, 200 ml. of water, and 150 ml. of concentrated hydrochloric acid.

For the determination, mix 1 ml. of 0.03% 1-nitroso-2-naphthol in 0.12% sodium hydroxide solution with 2 ml. of sample solution and 5 ml. of promoter solution. Heat at 40° for 15 minutes and cool. Read at 510 m μ .

By phosphotungstic-phosphomolybdic acid. Collagen and gelatine. For the preparation of the reagent, see Volume III, page 116. Hydrolyze the sample in 0.4% sodium hydroxide at 100° for 10 minutes. Cool, and dilute with 4 volumes of water. Mix 4.1 ml. of sample, 0.5 ml. of 20% sodium carbonate solution, and 0.4 ml. of the reagent. Heat at 37° for 40 minutes and cool. After 10 minutes, read at 750 m μ .

TRYPTOPHAN, 2-INDOYL-1-AMINOPROPIONIC ACID

Tryptophan is treated with an oxidizing agent and acetic acid in an anhydrous medium. Oxidation is performed by persulfate or hydrogen peroxide in the case of protein samples. The anhydrous conditions are maintained with sulfuric acid, and the rate of oxidation is controlled by thioglycolic acid.¹¹⁶ The oxidized solution is read at 550 m μ , and Beer's law is followed for 0.005-0.05 mg. of tryptophan. Large concentrations of phenol interfere. A closely related method uses ferric ion as the oxidizing agent.¹¹⁷ There is no interference by chloride, phosphate, glucose, cysteine, glutathione, or uric acid.

Tryptophanase catalyzes the quantitative conversion of L-tryptophan to indole. The indole formed is extracted with toluene and colorimetrically determined by *p*-dimethylaminobenzaldehyde in ethanol.¹¹⁸ Pyridoxal phosphate is the coenzyme of tryptophanase and is added to complete the enzyme.

¹¹⁶ Joseph Fischl, *J. Biol. Chem.* **235**, 999-1001 (1960); A. S. Ingelis and I. H. Leaver, *Anal. Biochem.* **7**, 10-17 (1964).

¹¹⁷ J. Opieńska-Blauth, M. Chareziński, and H. Bereć, *Ann. Biochem.* **6**, 69-76 (1963).

¹¹⁸ H. Frank and R. D. DeMoss, *Arch. Biochem. Biophys.* **67**, 381-97 (1957); F. A. Scott, *Biochem. J.* **80**, 462-4 (1961).

The reaction of tryptophan with glucose produces a fluorescence, which is read at 425 m μ .¹¹⁹ Separation from other amino acids is accomplished by passage through Dowex 50, sodium form. The fluorescence is quenched above pH 7. Beer's law is followed up to 0.02 mg. of tryptophan.¹²⁰ The fluorescence of tryptophan increases in duration but decreases in intensity with decrease in temperature.¹²¹ The presence of 48% of urea causes a shift in the fluorescence of tyrosine and tryptophan toward the red.¹²²

For tryptophan by L-aminooxidase and catalase, see page 254.

Of all the amino acids found in proteins, only tyrosine and tryptophan exhibit detectable fluorescence in aqueous media. At pH 11, the intensity of the fluorescence of tryptophan is approximately 100 times greater than that of an equivalent amount of tyrosine.¹²³

By use of a spectrophotometer capable of continuous activation of solutions and measurement of resultant fluorescence throughout the quartz-ultraviolet and visible regions of the spectrum, tryptophan is measured in the presence of excess tyrosine. Tyrosine is then determined after tryptophan is destroyed by acid hydrolysis. Under certain conditions, 5-methyltryptophan, 4-methyltryptophan and 5-hydroxytryptophan will react with the enzyme. Anthranilic acid does not interfere. Indole-acetic acid, 3-hydroxyanthranilic acid, tryptamine, isatin, oxindole, kynurenine, 5-hydroxytryptamine, skatole, and anthranilic acid do not interfere when present in tenfold excess to L-tryptophan. D-Tryptophan does not interfere. Casamino acids do not interfere. Other enzymes do interfere.

Tryptophan is measured by the purple color formed with *p*-toluene-sulfonic acid reagent.¹²⁴ Albumins do not react with the reagent and it is therefore not necessary to separate them from the sample. Tryptophan-containing proteins including fibrogen, casein, thrombin, edestin, and globulins, cerebrospinal fluid, jackbean meal, or crude pepsin yield the same color with the reagent. Beer's law is followed up to 4 mg. of hydrolyzed protein and the absorption maximum is between 550 and 560 m μ . This method is not applicable to free tryptophan but may be used for

¹¹⁹ Gerald D. Miller, John A. Johnson and Byron S. Miller, *Anal. Chem.* **28**, 884-7 (1956).

¹²⁰ Audrey White, *Biochem. J.* **71**, 217-20 (1959).

¹²¹ G. N. Barenboim, *Biofizika* **7**, 227-32 (1962).

¹²² Yu. A. Vladimirov and C.-K. Li, *ibid.* **7**, 270-80 (1962).

¹²³ Daniel E. Duggan and Sidney Udenfriend, *J. Biol. Chem.* **223**, 313-19 (1956).

¹²⁴ Ronald L. Searcy, *Arch. Biochem. Biophys.* **81**, 275-6 (1959).

protein tryptophan.¹²⁵ Erratic results are produced if any acetylation procedure is used before or during tryptophan determination. Different results are also obtained if the protein hydrolyzates are compared with the protein before hydrolysis.

The blue color formed by the reaction of tryptophan and *p*-dimethylaminobenzaldehyde (Vol. IV, p. 128) is accelerated by heating or by addition of an oxidizing agent. With these conditions, the color is unstable. The acid hydrolyzate of casein is added to stabilize the color. Methionine also stabilizes the color.¹²⁶ A variation of this method permits determination of indole, tryptophan, and anthranilic acid in the presence of each other.¹²⁷ Anthranilic acid and indole form colored products with *p*-dimethylaminobenzaldehyde in 0.5 *N* acid. Tryptophan does not react until the acid is 11 *N*.

A "pseudo" glyoxylic acid reagent is prepared with sodium hydroxide and chloral. The oxidizing agent for the reaction is sulfuric acid.¹²⁸ An alternative agent for development is tri(hydroxymethyl)aminomethane at pH 9.2 with trypsin added.¹²⁹

In potato juice, tryptophan is precipitated by mercuric sulfate but the tyrosine is not.¹³⁰ The filtered mercuric complex is dissolved in potassium cyanide solution. Thereafter, both tryptophan and tyrosine are determinable in their respective solutions. Treatment of solutions containing tryptophan and tyrosine with sodium nitrite and acetic acid causes a new maxima for tryptophan at 310 *mμ* and 270 *mμ* after 1.5 hours at 80°. ¹³¹ Tyrosine shows new maxima at 390 *mμ* and 265 *mμ*. Phenylalanine shifts to 350 *mμ*.

Tryptophan is determined in wool by hydrolyzing for 2 hours at 70° in a sealed tube with concentrated hydrochloric acid containing *p*-dimethylaminobenzaldehyde.¹³² By hydrolysis at 121°, the time for tryptophan in wheat flour is cut from 12 hours to 20 minutes.¹³³ When reacted with *N*-bromosuccinamide in a buffer for pH 7.4, only tryptophan and histidine

¹²⁵ D. F. Evered, *Anal. Biochem.* **2**, 280-1 (1961).

¹²⁶ J. C. Sanahuja, *Anales bromatol.* **7**, 25-34 (1955); Shotaro Nakajima and Genichi Okuyama, *J. Pharm. Soc. Japan* **76**, 620-4 (1956).

¹²⁷ D. Kupfer and D. E. Atkinson, *Anal. Biochem.* **8**, 82-94 (1964).

¹²⁸ C. Dumazert and R. Grangaud, *Trav. soc. pharm. Montpellier* **14**, 146-51; *Bull. soc. pharm. Marseille*, No. **10**, 97-102 (1954).

¹²⁹ P. M. Harrison and T. Hofmann, *Biochem. J.* **80**, 30P (1961).

¹³⁰ Th. Breyhan, *Z. anal. Chem.* **143**, 241-4 (1954).

¹³¹ V. Ya. Brodskii and I. M. Limarenko, *Doklady Akad. Nauk. SSSR* **95**, 313-16 (1954).

¹³² P. Miro, *Melliand Textilber.* **43**, 405-9 (1962).

¹³³ M. Gruener and M. Filajdic, *Agron. Glasnik* **11**, No. 7-9, 51-2 (1961).

fluoresce.¹³⁴ Tryptophan is also determined as the peptide value by glyoxylic acid and copper sulfate.¹³⁵

Another method is based on the shift of the absorption maximum of tryptophan from 280 $m\mu$ to 250 $m\mu$ by reaction with *N*-bromosuccinamide in dilute acid solution. The tryptophan must be in the peptide form rather than free.¹³⁶ This is applicable in the presence of appreciable amounts of haemoglobin, sterols, lipids, or polysaccharides. It is accurate to $\pm 5\%$ for casein, peptone, albumin, or trypsin. Tryptophan modifies the reaction of anthrone on carbohydrate. This is used for its determination in a mixture of the two.¹³⁷

Procedure—By fluorescence. Protein. Dissolve a 20-50-mg. sample in 1 ml. of 20% sodium hydroxide solution. Heat in an open tube in an autoclave for 20 hours at 2 atmospheres. Correct the sample for the moisture content as determined by drying a separate sample *in vacuo* at 100°. Cool, acidify with 1.5 ml. of 1:6 sulfuric acid, and dilute to 25 ml. with water. Clear by centrifuging.

To separate tryptophan from the other amino acids, use a 10 \times 300-mm. chromatographic column with ground joints and coarse sintered glass plates.¹³⁸ Jacket the tubes to permit temperature control by circulation of water from a constant temperature bath. The jacket is 108 cm. long and is made from 2-cm. tubing. The inlet should be about 2 cm. from the bottom of the jacket and the outlet about 4 cm. from the top. Fasten the jacket to the chromatographic tube with rubber stoppers.

Wash 1 pound of 250-500 mesh Dowex-50 hydrogen form with 1:2 hydrochloric acid on a funnel with gentle suction, using 4-8 liters of acid. The filtrate should be nearly colorless. Wash twice with water and wash with 8% sodium hydroxide solution until the filtrate is alkaline. Suspend the resulting sodium salt of the resin in 3 times its volume of 4% sodium hydroxide solution and heat at 100° for 3 hours with occasional shaking. Decant the supernatant liquid after 30 minutes of settling and replace with fresh hot 4% sodium hydroxide solution. Repeat the heating and replacement of sodium hydroxide 4 times. Filter, wash the resin free of alkali, pass through a 120-mesh screen with 6-8 liters of water, and filter.

¹³⁴ L. Brand and S. Shaltiel, *Biochim. Biophys. Acta* **75**, 145-8 (1963).

¹³⁵ C. H. Breiskorn and H.-W. Berg, *Z. Lebensmitt. Untersuch.* **109**, 302-6 (1959); *ibid.* **123**, 195-200 (1963).

¹³⁶ C. B. Breiskorn and J. Scheick, *Z. Lebensmitt. Untersuch.* **114**, 473-5 (1961).

¹³⁷ Y. Nakamura, *J. Pharm. Soc. Japan*, **81**, 846-50 (1961); Y. Nakamura, *J. Pharm. Soc. Japan*, **82**, 689-95 (1962).

¹³⁸ S. Moore and W. Stein, *J. Biol. Chem.* **192**, 663-81 (1951).

Prepare a sodium citrate buffer at pH 3.4. Dissolve 21 grams of citric acid monohydrate in 200 ml. of 4% sodium hydroxide solution and dilute to 500 ml. Add 110 ml. of 1:10 hydrochloric acid, 390 ml. of water, and 0.5 ml. of thiodiglycol per 100 ml.

Wash the resin on a filter with a small amount of this buffer and suspend in 2 volumes of the buffer. The thickness of the slurry should be such that, when settled, about 200 ml. of supernatant buffer is present over 100 ml. of settled resin. Remove all bubbles from the slurry by gentle stirring after standing for 1-2 hours. Pour the slurry into the column in 5 portions to obtain uniformity. Pour each portion through a funnel, bending the tip to direct the stream against the side of the tube. Allow the first portion of resin to settle under air pressure of 10-15 cm. of mercury until the level of resin is constant. Maintain the pressure until the liquid falls to within 10 cm. of the surface of the resin. Add the second portion of slurry and repeat until the final height of resin is 100 cm.

To prepare sodium citrate buffer at pH 5, mix 21 grams of citric acid monohydrate with 200 ml. of 4% sodium hydroxide solution and dilute to 500 ml. Dilute to 1 liter and add 1 gram of disodium Versenate and 15 ml. of benzyl alcohol. To prepare a phosphate buffer for pH 6.8, mix 500 ml. of 1.4% disodium phosphate solution, 450 ml. of 1.4% monosodium phosphate monohydrate solution, 1 gram of disodium Versenate, and 15 ml. of benzyl alcohol.

Mix a 2-ml. aliquot of hydrolyzate, containing a maximum of 6 mg. of amino acids, at pH 4, and 2 ml. of sodium citrate buffer at pH 5, and add to the column. Elute the extraneous amino acids with 45 to 50 ml. of citrate buffer for pH 5. Elute the tryptophan with 40-50 ml. of phosphate buffer for pH 6.8. Adjust the eluate of tryptophan to pH 1.38 and dilute to 50 ml.

To prepare the sodium citrate-hydrochloric acid buffer for pH 1.8, mix 20 parts of 25% sodium citrate solution and 60 parts of 1:10 hydrochloric acid. To prepare the sodium citrate-hydrochloric acid buffer for pH 1.38, mix 20 parts of 25% sodium citrate solution and 62 parts of 1:10 hydrochloric acid. Mix three aliquots of phosphate buffer containing up to 0.02 mg. of tryptophan with 5 ml. of sodium citrate-hydrochloric acid buffer at pH 1.38 containing 0.8 gram of glucose. Dilute, if necessary, to 10 ml. with phosphate buffer adjusted to pH 1.38. Heat in an autoclave for 4 hours at 118° and cool. Dilute 2-ml. aliquots to 25 ml. with sodium citrate-hydrochloric acid buffer at pH 1.8. Activate at 365 $m\mu$ and read at 425 $m\mu$ against a 0.1 ppm. sodium fluorescein solution.

Use a special spectrophotometer.¹³⁹ Dilute an 8-ml. aliquot of the acidified alkaline protein hydrolysate to 50 ml. with 5% sodium carbonate solution, so that the final solution contains 0.133-0.333 mg. of protein per ml. Activate at 280 $m\mu$ and read the fluorescence at 360 $m\mu$.

Plasma. Dilute a 1-ml. sample with 4 ml. of water and acidify with 0.5 ml. of 1:59 sulfuric acid. Precipitate the protein by addition with constant shaking of 0.5 ml. of 10% sodium tungstate solution and remove the precipitate by centrifuging. Add a 3-ml. aliquot of the supernatant liquid to 1 ml. of 5.2% barium chloride solution and remove the precipitate of barium sulfate and barium tungstate by centrifuging.

Treat a 2-ml. portion of the supernatant liquid with 0.5 ml. of 21% sodium carbonate solution to precipitate excess barium and adjust the pH to 11. Centrifuge at 3000 rpm. for 10 minutes. Activate a 1-1.5-ml. portion of the supernatant liquid at 280 $m\mu$ and read the tryptophan at 360 $m\mu$. As a blank, carry a water sample through the entire procedure.

By oxidizing agent and acetic acid. To prepare the potassium persulfate solution, add 100 ml. of water to 8 grams of potassium persulfate and shake for 3 minutes. Keep cold and use the supernatant liquid.

To a sample containing 0.005-0.05 mg. of tryptophan in 0.5 ml., add 2 ml. of glacial acetic acid. Mix, and add 1 ml. of concentrated sulfuric acid. Mix, and add 1 drop of potassium persulfate reagent and 1 drop of 5% thioglycolic acid solution in glacial acetic acid. Mix, and read after 10-15 minutes at 530-550 $m\mu$.

By tryptophanase. Indirectly as indole. To prepare the enzyme prepare a growth medium containing 8 grams of dibasic potassium phosphate, 0.05 gram of ammonium sulfate, 0.5 gram of ammonium chloride, 0.1 gram of magnesium chloride, 2 grams of succinic acid, and 0.5 gram of DL-tryptophan per liter. Adjust the pH to 6.9 before sterilization. Maintain *Escherichia coli*, strain Crookes, on nutrient agar slants stored at 4°. Autoclave 100 ml. of growth medium at 120° for 15 minutes. Inoculate from the slant, shake at room temperature for 36 hours, and use the entire culture as the inoculum for a carboy. The carboy contains 8 liters of medium fitted with an aerating tube, previously autoclaved at 120° for 1 hour.

Incubate the large culture for 48 hours at room temperature with vigorous aeration. Harvest the cells in a refrigerated supercentrifuge. Wash once with water and resuspend in 40-50 ml. of water. Subject the

* R. L. Bowman, P. A. Caulfield and S. Udenfriend, *Science* 122, 32 (1955).

suspension to sonic vibration using a 10-ke. oscillator operating at 0.9 amp. output for 45 minutes. Centrifuge to remove debris. Dialyze the opalescent fluid for 24 hours at 4° against 6 liters of water layered with 20-30 ml. of toluene. Agitate the water without disturbing the integrity of the toluene layer. After dialysis, test 1 ml. of enzyme for indole. If indole is present, repeat the dialysis. The dialyzed extract is used as the enzyme. Store at -20°. One unit of enzyme will produce 1 micromole of indole under the conditions of the procedure.

Mix 0.5 ml. of phosphate buffer at pH 7.55, 0.04 mg. of pyridoxal phosphate, and 50 units of enzyme. Dilute to 2.3 ml. with water. Incubate at 37° for 20-40 minutes. Add the mixture to 1 ml. of sample containing 0.1-1 micromole of L-tryptophan. Add 4 ml. of toluene, stopper, and shake at 37° for 40 minutes. To 1 ml. of the toluene layer containing 0.01-0.17 micromole of indole, add 1 ml. of 5% *p*-dimethylaminobenzaldehyde solution in 95% ethanol and 8 ml. of the acid-alcohol mixture prepared by adding 80 ml. of concentrated sulfuric acid to 1 liter of 95% ethanol. Mix, and read at 540 m μ after 10-15 minutes against a reagent blank.

By pseudoglyoxylic acid. To prepare the pseudoglyoxylic acid reagent, add 30.2 ml. of 4% sodium hydroxide solution to 10 grams of chloral dissolved in 60 ml. of water. Dilute to 100 ml. with water, let stand 4 hours and filter.

General. Cool 10 ml. of a solution containing 750 ml. of concentrated sulfuric acid and 0.1 gram of cupric sulfate pentahydrate per liter in ice. Add 0.5 ml. of the reagent to 2 ml. of a solution containing 0.05-0.25 mg. tryptophan. Heat for 30 minutes at 100° and read at 550 m μ .

Serum, or a precipitation of serum total globulins and serum γ -globulins. Dilute a 0.1-4 mg. sample of serum or dilute the precipitate to 10 ml. with 12% *p*-toluenesulfonic acid solution in glacial acetic acid. Incubate at 100° for 15 minutes. Cool and read at 560 m μ .

By ferric chloride. To 1 ml. of sample containing 0.002-0.04 mg. of tryptophan, add 2 ml. of a reagent containing 0.27 gram of ferric chloride hexahydrate dissolved in 0.5 ml. of water and diluted to a liter with glacial acetic acid. Add 2 ml. of concentrated sulfuric acid. Mix, and after 15 minutes read at 545 m μ .

By N-bromosuccinamide. Heat a sample of 0.1-0.3 gram with 10 ml. of 4% sodium hydroxide solution at 100° for 30 minutes. Cool, and acid-

ify with 6 ml. of 1:17 sulfuric acid. Dilute to 250 ml. with 1:350 sulfuric acid. Treat 10-ml. aliquots with amounts of 0.1% aqueous *N*-bromosuccinamide in the range of 0.1-1.0 ml. After 10 minutes, read at 280 $m\mu$. Compare the maximum value obtained with a graph prepared with the glycyl dipeptide of tryptophan. The result is linear for 1-5 mg. of tryptophan per 100 ml.

Indole, tryptophan, and anthranilic acid by p-dimethylaminobenzaldehyde. To 5 ml. of sample, add 1 ml. of 6% *p*-dimethylaminobenzaldehyde in 1:10 sulfuric acid. After 20 minutes, read the anthranilic acid at 420 $m\mu$, the indole at 550 $m\mu$. Chill, and add 3 ml. of concentrated sulfuric acid. After 3 hours, add 0.1 ml. of 0.1% sodium nitrite solution. After 30 minutes, read tryptophan at 580 $m\mu$.

5-HYDROXYTRYPTOPHAN

Using a method similar to that for the determination of serotonin, it is possible to read the fluorescence of 5-hydroxytryptophan. At an acidic pH *tert*-amyl alcohol extracts serotonin, *N*-acetylserotonin, 5-methoxytryptophan, bufotenine, and 5-hydroxytryptophan. An alkaline wash with *tert*-amyl alcohol takes up these other indoles and leaves behind all but 5% of the 5-hydroxytryptophan¹⁴⁰ (see page 157).

Procedure—Follow the procedure for serotonin, starting at the beginning and ending with "In another centrifuge tube, combine 8 ml. of heptane and 0.5 ml. 1:110 hydrochloric acid, 0.5% ascorbic acid," substituting *tert*-amyl alcohol for diethyl ether.

To the tube containing the sample, add 0.25 ml. of buffer for pH 10 saturated with sodium chloride and *tert*-amyl alcohol. Immediately shake for 1 minute, centrifuge for 1 minute, and discard the alcohol phase. Add another 3 ml. of *tert*-amyl alcohol, shake for 1 minute, centrifuge for 1 minute, and discard the alcohol. Add 0.2 ml. of 1:110 hydrochloric acid, mix, and add 3 ml. of *tert*-amyl alcohol. Shake 1 minute, centrifuge for 1 minute, and transfer the alcohol phase to the tube containing the heptane. Shake for 2 minutes and centrifuge. Measure the volume of the aqueous phase and read the fluorescence at 540-550 $m\mu$ with activation at 295 $m\mu$.

¹⁴⁰ W. B. Quay, *Anal. Biochem.* **5**, 51-9 (1963).

HISTIDINE, β -IMIDAZOLYL- α -ALANINE, 2-IMIDAZOLYL-1-AMINOPROPIONIC ACID

The reaction of histidine with iodine and pyridine produces a violet color read at 560 m μ .¹⁴¹ Histamine and histidine methyl ester give the same reaction. *N*-Acetylhistidine, imidazole and carnosine or *N*- β -alanylhistidine do not give the characteristic color. The following do not react with the reagent: benzimidazole, tryptophan, *N*-acetyltryptophan, indoleacetic acid, creatine, β -alanine, β -phenylalanine, tyrosine, sarcosine, β -mercaptoethylamine, ascorbic acid, 3-amino-1,2,4-triazole, and xanthine. For the microdetermination of histidine and histamine in protein hydrolysates, the histidine and histamine are adsorbed on an ion-exchange resin. Copper, zinc, iron, and thiocyanate reduce the color intensity. As little as 0.001 mg. per ml. can be detected. Beer's law is followed up to 0.06 mg. per ml.

The reaction of histidine with *p*-nitrobenzoyl chloride in acetone solution yields 1,2-di(*p*-nitrobenzamido)ethylene.¹⁴² When read at 417 m μ the sensitivity is 0.025 micromole or 0.004 mg. The concentration of acetone is critical. The method is also applicable to imidazole and histamine. The following compounds do not interfere: 1-methylimidazole, 4- or 5-bromoimidazole, 4- or 5-chloromethylimidazole, 4- or 5-hydroxymethylimidazole, 4- or 5-imidazolecarboxyaldehyde, 4- or 5-imidazolecarboxylic acid, the methyl ester of 4- or 5-imidazolecarboxylic acid, 2,4,5-tribromoimidazole, 2,4,5-trimethylimidazole, pilocarpine, ammonia, phenol, and pyridine.

1-Amino acid oxidase reacts with histidine at pH 7.8 in the presence of borate ions.¹⁴³ The product is an enol-borate complex of imidazolylpyruvic acid. After 30 minutes, this can be read at 292 m μ . For correction for other aromatic amino acids, repeat the determination at pH 6.5, at which histidine does not react.

The method by diazotized monochloroaniline (Vol. IV, p. 142) is improved by separation and concentration of the histidine by paper chromatography.¹⁴⁴ Histidine is determined by the biuret reaction in the

¹⁴¹ P. M. Newman and J. H. Turnbull, *Biochem. J.* **74**, 379-82 (1960).

¹⁴² Robert W. Cowgill, *Anal. Chem.* **27**, 1521-3 (1955).

¹⁴³ R. C. Balldridge and N. Greenberg, *J. Lab. Clin. Med.* **61**, 700-7 (1963).

¹⁴⁴ Hermann Frank and Heinrich Petersen, *Hoppe-Seyler's Z. physiol. Chem.* **303**, 276-81 (1956).

presence of oxygen.¹⁴⁵ When reacted with *N*-bromosuccinamide in a buffer for pH 7.4, only histidine and tryptophan fluoresce.¹⁴⁶

Procedure—*By pyridine and iodine.* To prepare the potassium triiodide, dissolve 0.075 gram of iodine and 0.4 gram of potassium iodide in 10 ml. of water. The pyridine used as a reagent is fractionally distilled to avoid erratic results.

To a 3-ml. solution containing 0.001-0.055 mg. of histidine, add 1-2 ml. of pyridine and adjust the pH to 11-12 with 0.8% sodium hydroxide solution. Dilute to 4-5 ml. with water and add 0.04 ml. of potassium triiodide reagent solution. Shake, and let stand for 30 seconds from the time the reagent was added. To stop further color development, add 0.5 ml. of 0.24% sodium dithionite solution to react with excess iodine. Mix, dilute to 5 ml. with water, and read at 560 $m\mu$ from 5 to 15 minutes after color development.

By p-nitrobenzoyl chloride. Adjust the volume of the sample to 1 ml. with water. Add 0.1 ml. of 8.4% sodium bicarbonate solution and 5 ml. of 1.1% *p*-nitrobenzoyl chloride solution in acetone. Swirl vigorously. After 10 minutes, add 2 ml. of 4% sodium hydroxide solution. Swirl, and let stand for 5 minutes. Dilute to 10 ml. with water and read after 30 minutes at 420 $m\mu$.

VALINE, α -AMINOISOVALERIC ACID

Valine is dinitrophenylated and the derivative separated on a chromatographic column. It is then read in the ultraviolet.¹⁴⁷ Carry out all operations concerning dinitrophenyl derivatives in artificial light. A 100-watt tungsten filament bulb is suitable but fluorescent light is to be avoided.

Procedure—Neutralize any nonvolatile acid with sodium bicarbonate. Evaporate a sample containing 0.1-0.18 mg. of valine to dryness. Take up the residue in 0.2 ml. of 10% sodium bicarbonate solution. Add 0.4 ml. of fresh 10% fluorodinitrobenzene in ethanol. Shake gently for 2 hours. Evaporate the ethanol *in vacuo*. Add 2.5 ml. of water and 5 ml. of chloroform. Shake vigorously. Separate the aqueous layer and further extract

¹⁴⁵ Stuart S. Updike and Alexander L. Dounce, *Anal. Biochem.* **6**, 1-12 (1963).

¹⁴⁶ L. Brand and S. Shaltiel, *Biochim. Biophys. Acta* **75**, 145-8 (1963).

¹⁴⁷ N. A. Matheson, *Biochem. J.* **88**, 146-55 (1963).

the chloroform layer with 2.5, 2.5, and 2.5 ml. of water. Combine the aqueous extracts and add 0.2 ml. of 1:20 hydrochloric acid. Extract with three 3-ml. portions of ether previously washed with 5% ferrous sulfate to make it peroxide-free. Evaporate the ether extracts to dryness.

Take up in an appropriate volume of ethyl acetate saturated with water. Prepare a 40×1 -cm. chromatographic column. As a buffer for pH 8.3, mix 250 ml. of 0.2 *M* tris base with 200 ml. of 1:100 hydrochloric acid and dilute to 500 ml. For use, add 3.5 grams of sodium chloride to each 100 ml.

Mix 200 ml. of ethyl acetate with 40 ml. of the buffer and separate the phases. Slurry about 4 grams of Hyflo Supercel with 50 ml. of the upper phase. Add 2.5 ml. of the lower phase dropwise. Shake until free of lumps. Close the bottom of the chromatographic column and add enough of the top phase to cover the shoulder. Add a 1-cm. perforated silver disc and a 1-cm. paper disc. Pour about half of the suspension of Hyflo Supercel into the tube and agitate to remove lumps and air bubbles. Build up the column 1-2 mm. at a time with agitation, ending with a 15-19-cm. column.

Transfer the sample to the column in water-saturated ethyl acetate. Wash in with 0.2-ml. washes of top phase and fill the head space with the top phase. Allow to flow at about 1 ml. per minute. Chromatograph to separate the valine phase. Dinitrophenyl valine comes immediately behind the copious dinitrophenol phase. The dinitrophenol begins to emerge at 19 ml. of eluate and continues to 24 ml. Dinitrophenyl valine emerges at 26-31 ml.

As a buffer for pH 11, mix four parts of 0.142% solution of anhydrous disodium phosphate with one part of 0.38% solution of trisodium phosphate dodecahydrate.

To 12 ml. of eluate, add 20 ml. of buffer and keep at 30° for 10 minutes. Shake, and return to the bath until separate layers form. Read the lower layer at 363 $m\mu$ against a blank similarly treated.

p-HYDROXYPHENYLPYRUVIC ACID

For hydroxyphenylpyruvic acid (a metabolite of tyrosine) in urine, the sample is treated with lead acetate and hydrochloric acid. After centrifuging, it is passed through Dowex 50 W-X4. Thereafter, following a series of extractions it is read in butyl acetate at 275 $m\mu$.¹⁴⁸

¹⁴⁸ Ira J. Holcomb, D. S. McCann and A. J. Boyle, *Anal. Chem.* 37, 1657-9 (1965)

Procedure—Urine. To 10-25 ml., add 1 ml. of saturated lead acetate solution. Add 0.5 ml. of concentrated hydrochloric acid and centrifuge. Prewash a 6 \times 1-cm. column of 50-100 mesh Dowex 50 W-X4 successively with 25 ml. of 4% sodium hydroxide solution, 25 ml. of water, 25 ml. of 1:10 hydrochloric acid and 25 ml. of 1:1.5 hydrochloric acid. Pass the sample through the column. Elute with 1:20 hydrochloric acid collecting 80 ml. in 15-20 minutes. Extract the eluate with 40, 30, and 20 ml. of 1:1 *n*-butyl acetate-*n*-butyl alcohol. Extract the combined extracts with 10, 5, and 5 ml. of 4% sodium hydroxide solution. Adjust the pH of these combined extracts to 7.6 with 1:1 hydrochloric acid. Extract with 20, 10, and 10 ml. of butyl acetate. Adjust the combined extracts to 40 ml. and read at 275 $m\mu$.

β -SULFOALANINE, α -AMINO- β -SULFOPROPIONIC ACID, CYSTEIC ACID

Cysteic acid is separated from other amino acids by low voltage paper electrophoresis at pH 3.6. After the color reaction with ninhydrin in the presence of cupric ions, it is determined by direct photometry of the paper strip, or by elution with ethanol before reading.¹⁴⁹ As little as 0.05% of cysteic acid in proteins may be determined. This method is also applicable to the determination of cystine plus cysteine after oxidation to cysteic acid. Beer's law is followed for 0.004-0.02 mg. when the cysteic acid is eluted with ethanol. For direct photometry of the strips, the range is 0.004-0.012 mg.

Sample—Wool. Dry a 1-gram sample for 2 hours at 105°. Cool and weigh. Add 10 ml. of 1:1 hydrochloric acid, seal, and heat for 24 hours at 105°. Cool to room temperature, open, and evaporate *in vacuo*. Add 15 ml. of water to remove hydrochloric acid and evaporate *in vacuo*. Repeat with two additional 15-ml. portions of water. Dissolve the residue in a few ml. of water and dilute to 10 ml. with water. If the sample contains less than 0.2% of cysteic acid, repeat the hydrolysis and dilute the hydrolysate to only 5 ml. with water. Apply samples of 0.2, 1.0 and 2.0 ml. to the paper strip for unknown samples. If the concentration of cysteic acid is roughly known, apply a sample containing 0.004-0.02 mg. for the elution procedure and a sample containing 0.004-0.012 mg. for direct photometry.

¹⁴⁹ J. F. Diehl, *Anal. Chem.* **31**, 1204-6 (1959).

Procedure—To prepare the copper nitrate reagent, add 0.2 ml. of 1:9 nitric acid to 1 ml. of a saturated copper nitrate solution. Dilute to 100 ml. with 95% ethanol.¹⁵⁰ To prepare the buffer for pH 3.6, mix 1 volume of pyridine with 10 volumes of glacial acetic acid and 89 volumes of water. The electrophoresis apparatus consists of a Durrum-type paper electrophoresis cell, Spinco Model R, operated by a Spinco Duostat power supply. The cell accommodates eight paper strips 31.5 cm. long and 2.9 cm. wide. For the paper strips, Whatman 3-mm. paper is used.

For each run, mark 8 strips with a pencil and hang like an inverted V over the rack in the electrophoresis cell. Soak by pouring buffer over the strips along their apices and drain for 20 minutes. Evenly streak the cysteic acid solutions or protein hydrolysates across the paper strips at the apices. Apply 0.6-1 ml. of standard cysteic acid solution containing 1 mg. of cysteic acid per ml. to each of two strips, using the 6 remaining for the sample.

Allow electrophoresis to proceed for 2 hours at a constant current of 30 milliamperes. The voltage at the beginning of the run is about 280 volts and after 2 hours about 230 volts. This represents a voltage gradient of 8.9 and 7.3 volts per cm. respectively. Remove the rack with the strips. Extend the rack to bring the strips in a horizontal position and heat in an oven for 15 minutes at 105°.

Immerse the strips for 10 seconds in a solution containing 5 grams of ninhydrin in a mixture of 930 ml. of 1-butanol and 70 ml. of glacial acetic acid. Allow the excess liquid to drip off and dry the strips for 15 minutes at 60° in an oven. To achieve full color development, saturate the atmosphere in the oven with water or hold the strips over a steam bath for 30 seconds. Spray both sides of the strips with copper nitrate solution in ethanol, which changes the color from blue to pink and produces a more stable color.

Allow 10 minutes for the evaporation of the ethanol. Scan the cysteic acid bands of the two standard strips and the 6 sample strips in a balance-beam type of photoelectric recording photometer, Spinco Analytrol Model R. When the B-2 balancing cam is used, a record of the variations of absorbance along the strip is produced. The integrated areas under the density peaks are given in square centimeters $\times 10^{-1}$. Use a slit width of 1 mm. and 500-m μ interference filters. Prepare a standard curve by plotting the two known concentrations against the densities and drawing a straight line from the origin to the two points. Calculate the con-

¹⁵⁰ E. Kawerau and T. Wieland, *Nature* **168**, 77-78 (1951).

centration of cysteic acid on the other 6 strips by reference to the standard curve. Prepare a new standard curve for each determination, as the results are not always reproducible.

Alternatively, cut out the pink bands of cysteic acid, place in a test tube, and extract each with 4 ml. of 95% ethanol for 30 minutes, shaking frequently. To prepare the blank, cut out an unstained portion of the strip and extract similarly with ethanol. Read at 504 $m\mu$.

β -AMINOISOBUTYRIC ACID

β -Aminoisobutyric acid is developed with dinitrofluorobenzene. The derivative is extracted with ether and chromatographed on paper with heptane-pyridine. On elution with sodium bicarbonate solution, this is read at 360 $m\mu$.¹⁵¹ Dinitrophenyl derivatives of urinary amino acids do not interfere. It is also read at 480 $m\mu$ as the copper complex in ethanol.¹⁵²

Procedure—Urine. Dilute a sample 1:3 with 1% sodium bicarbonate solution. To 3 ml. of the diluted sample, add 0.9 ml. of 1% dinitrofluorobenzene solution in 95% ethanol. Cool, and maintain at 37° for 2 hours with frequent shaking. Extract excess reagent with three 10-ml. portions of ether. Acidify the aqueous phase with 0.3 ml. of 1:3 hydrochloric acid. Extract the developed color with three 10-ml. portions of ether and evaporate the combined extracts to dryness. Dissolve the residue in tetrahydrofuran and chromatograph on paper with 70:30 heptane-pyridine for 10-12 hours. Locate the spot with ultraviolet light and elute with one 15-ml. portion and one 10-ml. portion of 0.3% sodium bicarbonate solution. Read at 360 $m\mu$.

γ -AMINO BUTYRIC ACID

γ -Aminobutyric acid and basic amino acids are retained on Dowex 50 resin when most ninhydrin-reactive substances are eluted with a buffer at pH 3.1. The γ -aminobutyric acid is then eluted with a buffer at pH 5.1 and developed with ninhydrin.¹⁵³ Passage through Amberlite CG-50 removes interferences from basic amino acids.

γ -Aminobutyric acid, glutathione, glutamine, and glutamic acid are separable by column chromatography, after which, each is determined.

¹⁵¹ Georg B. Gerber and Gisela Gerber, *Clin. Chim. Acta* **5**, 607-8 (1960).

¹⁵² V. Jirgl and J. Sochman, *Clin. Chim. Acta* **7**, 388-91 (1962).

¹⁵³ Robert P. Sandman, *Anal. Biochem.* **3**, 158-63 (1962).

As the first step, the solution is passed through alumina which adsorbs glutamic acid and a glutathione-*p*-chloromercuriophenyl sulfonic acid complex. Glutamic and γ -aminobutyric acid are eluted and separated by passage through Dowex 50.¹⁵⁴

Thereafter, by reaction with nitrous acid, γ -aminobutyric acid forms γ -butyrolactone, which is developed with hydroxylamine hydrochloride and ferric chloride. Interference of large amounts of aspartic acid can be eliminated by heating the sample at 50° for 1 hour after addition of the glacial acetic acid and sodium nitrite reagent.

Procedure—Brain tissue. To prepare a sodium citrate buffer for pH 5, dissolve 21 grams of citric acid in water, add 200 ml. of 4% sodium hydroxide solution, and dilute to 500 ml. To prepare a sodium citrate buffer for pH 3.1, dilute 500 ml. of the pH 5 buffer with 110 ml. of 1:10 hydrochloric acid and 390 ml. of water. Add 0.5 ml. of thiodiglycol per 100 ml. Treat 200-400 mesh Dowex 50-X8 with alkali, and wash. Buffer to pH 3.1 with the citrate buffer.

Stir 1.5 kilos of Amberlite CG-50 with 3.5 liters of water for 20 minutes. Allow 30 minutes to settle, remove any foam on the surface, and withdraw the supernatant liquid. Repeat this procedure 4-5 times with 2-liter portions of water, or until the supernatant liquid is clear after 15 minutes of settling. Air dry and add the filter cake to 4 liters of acetone. Stir for 3 hours. Filter and wash with 8 liters of acetone until the filtrate is clear. Air dry and suspend the resin in water. Stir to eliminate bubbles, and remove the last traces of acetone by washing on a filter with 24 liters of water. Mix the resin with 5 liters of water.

Add 1400 ml. of 40% sodium hydroxide solution over a 30-minute interval. Stir until the heat evolution subsides, usually about 3 hours. Wash the sodium salt of the resin by decanting with five 2-liter portions of water. Wash on a filter with about 12 liters of water or until the pH of the filtrate is approximately 10. Convert to the acid form by washing with 10 liters of 1:3 hydrochloric acid over a period of 4 hours. Wash with 6 liters of water. Stir the acid form of the resin with 5 liters of buffer at pH 5. Adjust the pH with 30-ml. portions of 40% sodium hydroxide solution as neutralization occurs, until the pH remains constant for 15 minutes. Wash with 30 liters of buffer over a period of 8 hours, or until the pH of the final filtrate is the same as that of the buffer.¹⁵⁵ Prepare

¹⁵⁴ S. Berl and H. Waelsch, *J. Neurochem.* **3**, 161-9 (1958).

¹⁵⁵ C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.* **200**, 493-505 (1953).

10 \times 150-mm. glass columns by drawing out one end and adding a glass wool plug at the bottom.

Homogenize a 1.3-gram sample with the cerebellum removed with 6 ml. of water. Add 1 ml. of 10% trichloroacetic acid and centrifuge. Decant the supernatant liquid and wash the centrifuge tube and precipitate with several portions of water, adding the washings to the extract. Dilute to 25 ml. and neutralize. Use a 5-ml. aliquot containing 0.2-0.8 micromole of γ -aminobutyric acid.

Pour a slurry of Dowex 50 in citrate buffer at pH 3.1 into the column to form a 30-mm. column. Prepare a second column with 1 gram of Amberlite CG-50 suspended in water. Connect the Amberlite column to the Dowex column with a rubber stopper. Shake the neutralized sample with 1 gram of Amberlite CG-50 for 1 hour. Pour this slurry on top of the Amberlite column and wash three times with 5 ml. of water to remove the basic amino acids. Discard the eluate which has passed through both columns. Disconnect the two columns. Wash the Dowex column with two 5-ml. portions of citrate buffer at pH 3.1. Add citrate buffer at pH 5.1 and collect 10 ml. of eluate. Develop a 2-ml. aliquot with ninhydrin, following the procedure under "Amino Acids in General,"¹⁵⁶ page 222.

Brain tissue in the presence of glutamic acid, glutamine, and glutathione. To prepare the alumina, treat with twice its volume of 1:16 acetic acid. Wash repeatedly with water until the washings are neutral. Treat with twice its volume of 1:20 ammonium hydroxide. Wash until neutral and treat with twice its volume of 1:110 hydrochloric acid. Wash until neutral. Store the alumina under water. Fill a funnel with water and add the alumina suspension with a pipet. Pack the column by stirring the alumina with a fine wire and tapping the column until the alumina does not settle further. The final size of the column is 2 \times 0.3 cm.

To prepare an ammonium formate buffer for pH 3.1, dilute 375 ml. of 9% formic acid and 200 ml. of 1:20 ammonium hydroxide to 1 liter with water. To prepare ammonium acetate buffer for pH 5, dilute 147 ml. of 12% acetic acid and 200 ml. of 7.7% ammonium acetate solution to 1 liter with water.¹⁵⁷

Convert 400-mesh Dowex X4 to the sodium form and transfer back to the hydrogen form. Equilibrate the resin with ammonium formate buffer at pH 3.1 and store under an equal volume of buffer. Prepare a

¹⁵⁶ Stanford Moore and William H. Stein, *J. Biol. Chem.* **211**, 907-13 (1954).

¹⁵⁷ C. H. W. Hirs, Stanford Moore and William H. Stein, *ibid.* **195**, 669-83 (1952).

resin column 12×0.7 cm. by pouring the resin suspension into a tube with a tapered tip covered with a glass wool plug. Allow the resin to settle by gravity and adjust the final height to 12 cm. Wash the column with 5 ml. of ammonium formate buffer.

Extract a 100-200 mg. sample with four 1-ml. portions of cold 5% trichloroacetic acid solution. Centrifuge the suspension at 15,000 rpm. Neutralize the combined extracts with 8% sodium hydroxide solution. Add 0.5 ml. of 1.6% *p*-chloromercuriphenyl sulfonic acid solution to complex glutathione. Dilute to 6.2 ml. with water and adjust the pH to 7 with less than 0.01 ml. of *N* acid or base. Pass two 3-ml. aliquots through alumina columns and wash twice with 1 ml. of water, adding the washings to the effluent. The glutamic acid and glutathione-*p*-chloromercuriphenyl sulfonic acid complex remain in the column, and the glutamine and γ -aminobutyric acid are eluted. Elute glutamic acid with three 5-ml. portions of 3% acetic acid solution. Elute the glutathione complex with two 2-ml. portions of 1:39 ammonium hydroxide. To separate glutamine and γ -aminobutyric acid, adjust 5 ml. of the eluate from the alumina column to pH 2 with 0.25 ml. of 1:59 hydrochloric acid. Pass a 5-ml. aliquot through the Dowex column and wash the sides of the column down with two 0.5 ml. portions of 1:1100 hydrochloric acid. Elute the column with 12 ml. of ammonium formate buffer at pH 3.1. Collect fractions of 5-6, 6-8 and 8-9 ml. separately for the glutamine determination. Elute the column with 10 ml. of ammonium acetate buffer at pH 5 and collect fractions of 4-6, 6-8 and 8-10 ml. separately for γ -aminobutyric acid determination.

To the glutathione solution, add 0.035 ml. of glacial acetic acid. Add 0.05 ml. of sodium nitrite solution containing 80 grams in 100 ml. of water. Let stand at room temperature for 20 minutes. Add 0.25 ml. of 27% hydroxylamine hydrochloride solution. After 5 minutes, add 1.3 ml. of 4% sodium hydroxide solution. After 10 minutes, add 0.2 ml. of 3:1 hydrochloric acid and 1 ml. of 20% ferric chloride solution in 1:10 hydrochloric acid. Centrifuge for 10 minutes at 2000 rpm to precipitate the *p*-chloromercuriphenyl sulfonic acid. Read at $540 m\mu$ against 1 ml. of ferric chloride reagent in 2 ml. water and subtract the reagent blank.

To determine glutamic acid, follow the procedure for glutathione, starting at "Add 0.05 ml. of sodium nitrite solution . . ." and ending with ". . . and 1 ml. of 20% ferric chloride solution in 1:10 hydrochloric acid." Read at $540 m\mu$ against 1 ml. of ferric chloride reagent in 2 ml. of water and subtract a reagent blank.

To determine γ -aminobutyric acid, concentrate the 2 ml. of eluate to

0.5 ml. at 60° with the aid of a gentle stream of filtered air. Add 0.035 ml. of glacial acetic acid. Add 0.05 ml. of the sodium nitrite solution and let stand at room temperature for 2½ hours. Add 0.25 ml. of 27% hydroxylamine hydrochloride solution. After 5 minutes, add 1 ml. of 6.8% sodium hydroxide solution. After 10 minutes, acidify with 0.25 ml. of 3:1 hydrochloric acid and add 1 ml. of 20% ferric chloride solution in 1:10 hydrochloric acid. Read at 540 mμ against a mixture of 1 ml. of ferric chloride reagent and 2 ml. of water. Subtract a reagent blank. Calculate as follows:

Micromoles of glutamic acid per gram of brain tissue

$$= \frac{1000}{\text{mg. of tissue}} \times \frac{6.2}{3} \times \frac{15}{2} \times \text{reading} \times 0.73$$

Micromoles of glutamine per gram of brain tissue

$$= \frac{1000}{\text{mg. of tissue}} \times \frac{6.2}{3} \times \frac{5.25}{5} \times \frac{2}{0.5} \times \text{reading} \times 0.73 \times \frac{100}{95}$$

Micromoles of glutathione per gram of brain tissue

$$= \frac{1000}{\text{mg. of tissue}} \times \frac{6.2}{3} \times \text{reading} \times 0.73$$

Micromoles of γ-aminobutyric acid per gram of brain tissue

$$= \frac{1000}{\text{mg. of tissue}} \times \frac{6.2}{3} \times \frac{5.25}{5} \times \frac{0.73 \times 100}{73} \times \text{reading}$$

THREONINE, 2-HYDROXY-1-AMINOBTUTYRIC ACID

Periodate oxidation of threonine liberates acetaldehyde, which is measured *in situ* with dihydrodiphosphopyridine nucleotide and alcohol dehydrogenase¹⁵⁸ (cf. Vol. IV, pp. 145-6). Excess periodate is reduced with a mercaptan. Glyoxylate, a product of threonine oxidation, tartrate, and 2,3-butanediol interfere, while compounds that yield formaldehyde with periodate do not. The procedure is applicable to 0.01-0.1 micromole of threonine at 340 mμ. For determination as acetaldehyde in the presence of serine, see page 251.

Procedure—Adjust a 10% 3-mercaptopropionic acid solution to pH 6 with 5.6% potassium hydroxide solution. Store frozen and keep at 0° when in use. Replace weekly. Prepare a 0.0025 M solution of dihydrodi-

* Martin Flavin and Clarence Slaughter, *Anal. Chem.* 31, 1983-4 (1959).

phosphopyridine nucleotide solution and adjust the pH to 7.5. Store frozen and keep at 0° when in use. The yeast alcohol dehydrogenase is obtained as a suspension in ammonium sulfate of 60 mg. of protein per ml. Dilute 100:1 in the following mixture: 0.1% bovine serum albumin, 0.3% reduced neutral glutathione and 0.7% potassium pyrophosphate at pH 7.5. To a sample containing 0.02-0.1 micromole of threonine, add 0.1 ml. of *M* potassium phosphate buffer at pH 7.5 and dilute to 1 ml. with water. Add 0.02 ml. of 4% sodium metaperiodate solution. Mix well and let stand for 30 seconds. Reduce excess periodate with 0.03 ml. of 3-mercaptopropionic acid reagent and stir for 30 seconds. Add 0.04-0.05 ml. of dihydrodiphosphopyridine nucleotide reagent. Stir, and read twice at 340 m μ . If the reduction of periodate is complete, there will be no decrease in absorbance. Add 0.02-0.03 ml. of alcohol dehydrogenase and follow the oxidation of the nucleotide to acetaldehyde at 340 m μ until two successive readings at half-minute intervals show no decline in absorbance. The total decrease in absorbance corrected for dilution by the enzyme is the measure of the amount of threonine present.

GLUTAMIC ACID, 3-CARBOXY-1-AMINO BUTYRIC ACID

Glutamic acid in protein hydrolyzates is adsorbed with aspartic acid on Amberlite IR-4B columns at pH 3-4 and eluted with 1:10 hydrochloric acid. The eluates are treated with nitrite and the lactone formed is determined colorimetrically.¹⁵⁹

On autoclaving protein hydrolyzates at pH 3.3 for 4 hours at 125°, the glutamic acid is converted to pyrrolidone-carboxylic acid.¹⁶⁰ This is determined by treatment with hydroxylamine hydrochloride and ferric chloride. Beer's law is followed for 10-50 micromoles of pyrrolidonecarboxylic acid.

The determination by ninhydrin (Vol. IV, pp. 146-7) continues to be used. It requires standardization of time and temperature of heating.¹⁶¹ As determined in soups, the principal other amino acids that may be present are cystine and aspartic acid. Acetone is preferable to methyl Cellosolve as the solvent. As applied to hydrolysates of plant saps, the ninhydrin color is subjected to electrophoretic or chromatographic separation. Thereafter, the spots are extracted with methanol and read at 510 m μ .¹⁶²

¹⁵⁹ S. Kuk Meiri and N. Lichtenstein, *Anal. Chim. Acta* **14**, 266-8 (1956).

¹⁶⁰ Ada Zamir and N. Lichtenstein, *ibid.* **12**, 577-9 (1955).

¹⁶¹ Franco De Francesco, *Boll. lab. chim. provinciali* (Bologna) **8**, 202-3 (1957).

¹⁶² Albert Niemann, *Naturwissenschaften* **45**, 12 (1957).

For glutamic acid by hydroxylamine hydrochloride and ferric chloride in the presence of glutamine, glutathione, and γ -aminobutyric acid, see γ -aminobutyric acid, page 273.

L-Glutamic acid in culture media of *Lactobacillus arabinosus* is developed turbidimetrically by heating at 100° for 18 hours.¹⁶³

Sample—Protein. Reflux a 1.5-2 gram sample with 20 ml. of 1:1 hydrochloric acid in an oil bath at 120-125° for 35 hours. Add 1 gram of purified charcoal and continue heating for 1 hour. Cool, add 10 ml. of water, and filter. Wash the residue with 25 ml. of water. Adjust the pH of the filtrate to 3-4 with 28% sodium hydroxide solution and autoclave for 4 hours at 125°. Remove any precipitate by filtering, and wash. Dilute to 100 ml. with water. Develop as pyrrolidone-carboxylic acid.

Alternatively hydrolyze as above, through "Wash the residue with 25 ml. of water." Concentrate the filtrate *in vacuo*, add water and evaporate the solution *in vacuo*. Repeat the dilution and evaporation twice more. Dissolve the dry residue in 50 ml. of water. Adjust a 5-ml. aliquot to pH 3.5-4 with 14% sodium hydroxide solution and dilute to 15 ml. with water.

Treat 40-60 mesh Amberlite IR-4B resin with dilute hydrochloric acid and wash with water by decantation until the pH of the washings is 3-4. Transfer the resin to a tube of 1 cm. internal diameter to a height of 15 cm. Wash with water until the pH of the effluent is 3-4. Add 5 ml. of the sample to the top of the column. Adjust the flow rate to 1-2 ml. per minute. Pass the effluent through the column again and wash with 40 ml. of water or until a portion of the washings no longer gives a color reaction with ninhydrin.

Elute the adsorbed aminodicarboxylic acid with 100 ml. of 1:10 hydrochloric acid. Concentrate the eluate to dryness *in vacuo* and dissolve the residue in 25 ml. of water. Develop with nitrous acid.

Procedure—As pyrrolidone-carboxylic acid. To prepare the ferric chloride reagent, mix equal volumes of 1:5 hydrochloric acid, 15% trichloroacetic acid solution, and 5% ferric chloride solution in 1:110 hydrochloric acid.

To an 8-ml. aliquot of the autoclaved hydrolyzate, add 4 ml. of 50% hydroxylamine hydrochloride solution in 8% sodium hydroxide solution. Stopper with a rubber stopper containing a thick-walled capillary tube

¹⁶³ Tomoyuki Ishikura, Tadashi Sakamoto, Ichiya Kawasaki, Toshinas Tsumoda and Kikuko Narui, *Agric. Biol. Chem.* **28**, 700-9 (1964).

and heat for 15 minutes at 100°. Cool, and add 1.5 ml. of water to 1.5 ml. of the sample. Add 2.5 ml. of ferric chloride reagent and read immediately at 540 m μ against a mixture of 2.5 ml. of ferric chloride reagent and 3 ml. of water.

$$\text{per cent of glutamic acid} = \frac{a \times 1.14 \times 100,000}{b \times (100 - m) \times 93.5}$$

in which

a = milligrams of pyrrolidonecarboxylic acid found

b = grams of protein in sample

m = percentage of moisture estimated by drying the proteins *in vacuo* over phosphorus pentoxide at 100°

1.14 = the conversion factor from pyrrolidonecarboxylic acid to glutamic acid according to the ratio of the molecular weights

93.5 = average percentage value for ring closure of glutamic acid to pyrrolidonecarboxylic acid

By nitrous acid. To a 5-ml. aliquot, add 1 ml. of glacial acetic acid and 2 ml. of sodium nitrite solution containing 8 grams per 10 ml. of water. Maintain at 25° for 10 minutes. Place the sample in an ice bath and add 10 ml. of 14% hydroxylamine hydrochloride solution dropwise. Dilute to 25 ml. with water. Add a 3-ml. aliquot to a solution containing 1 ml. of 14% hydroxylamine hydrochloride solution and 2 ml. of 14% sodium hydroxide solution. After 4 minutes, add 1.65 ml. of 1:2 hydrochloric acid to adjust the pH to 1-1.2 and dilute to 10 ml. with 6% ferric chloride solution in 1:110 hydrochloric acid. Read the color immediately at 540 m μ .

METHIONINE, α -AMINO- γ -METHYLMERCAPTOBUTYRIC ACID

Methionine can be measured by reaction with ninhydrin.¹⁶⁴ The sensitivity of the reaction is 0.5×10^{-6} gram per ml. Upon acidification of an alkaline solution of methionine or radioactive methionine, methyl mercaptan is formed.¹⁶⁵ This gives a stable red color with sodium nitroprusside. The sensitivity is 15×10^{-6} gram per ml. Tryptophan, which inter-

¹⁶⁴ I. N. Bukharov and V. V. Agapov, *Metody Polucheniya i Izmeren. Radioaktiv. Preparatov, Sbornik Statci* 1960, 211-16; H. Mikulowska and W. Slowinski, *Proc. Inst. Lab. Badawczych Przemyslu Spozywczego* 13, 133-40 (1963).

¹⁶⁵ M. Sterescu and Ruxandra Simionovici, *Rev. chim.* 7, 299-301 (1956); S. K. Shrivastava, R. B. Chakravarty, and G. K. Ray, *Indian J. Pharm.* 25, 97-8 (1963).

feres, is removed with activated carbon. Cystine and cysteine do not interfere.

Procedure—*By ninhydrin.* Dilute a 0.1-0.5 ml. sample to 0.5 ml. with water. Dilute 2 ml. of a 0.5% sodium cyanide solution to 100 ml. with pyridine. To the sample, add 1 ml. of pyridine solution of sodium cyanide, 1 ml. of 80% phenol solution in 95% ethanol, and 0.2 ml. of 5% ninhydrin in 95% ethanol. Heat for 5 minutes at 100°. Cool for 5 to 7 minutes and dilute to 10 ml. with 60% ethanol. Read at 570 $m\mu$ against water.

By sodium nitroprusside. To 3-10 grams of protein hydrolysate, add 150 mg. of charcoal. After shaking, set aside for 30 minutes. Filter, and wash the filter with 1:35 hydrochloric acid. Adjust the pH of the filtrate and washings to 3.5-4 with 20% sodium hydroxide solution and dilute to 50 ml.

To a 5-ml. aliquot, add 1 ml. of 57% sodium hydroxide solution and 0.6 ml. of freshly prepared 10% aqueous sodium nitroprusside. After 10 minutes at 35-40°, cool. Add 5 ml. of a 9:1 mixture of concentrated hydrochloric acid and 85% phosphoric acid. After shaking, let stand at room temperature for 10 minutes and read at 530 $m\mu$.

GLUTATHIONE

Glutathione is a material that introduces problems of classification, since, as a tripeptide, it could be called a simple protein. It has been covered in Volume III, pages 489-93, and in Volume IIIA, pages 460-1, as a sulfur compound. It also appears rather casually in Volume IV, page 106, as an amino acid. A reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) merits its inclusion here as an amino acid.¹⁶⁶ The temperature effect is insignificant. Color is developed immediately, does not fade in 5 minutes, and the loss after 10 minutes is under 2%. The reaction should be carried out at pH 5.7-8.3. The technic specified will give 7.7. Other sulfhydryl compounds interfere.

The reaction of sodium nitroprusside with mercapto groups is applicable to reduced glutathione.¹⁶⁷ In the reduced form, it is determinable

¹⁶⁶ T. D. Stevenson, B. L. McDonald and S. Roston, *J. Lab. Clin. Med.* **56**, 157-60 (1960); J. C. Kaplan and J. C. Dreyfus, *Bull. Soc. Chim. biol.* **46**, 775-83 (1964).

¹⁶⁷ E. Mortensen, *Scand. J. Clin. Lab. Invest.* **16**, 87-97 (1964).

enzymatically.¹⁶⁸ For glutathione by hydroxylamine and ferric chloride in the presence of glutamine, glutamic acid, and γ -aminobutyric acid, see page 275.

Procedure—*Blood*. By 5,5-dithiobis-(2-nitrobenzoic acid). As a precipitating solution, dissolve 1.67 gram of metaphosphoric acid, 0.2 gram of disodium ethylenediaminetetraacetate, and 30 grams of sodium chloride in 100 ml. of water. Store at 4°. It is stable for about 3 weeks. A fine precipitate that may form is essentially ethylenediaminetetraacetate and does not interfere.

Mix 0.2 ml. of blood with 1.8 ml. of water. Add 3 ml. of the precipitating solution. Let stand for 5 minutes and filter. Prepare a 4.25% solution of anhydrous disodium phosphate. Mix 2 ml. of the sample filtrate with 8 ml. of this phosphate solution. Add 1 ml. of a 0.04% solution of 5,5'-dithiobis-(2-nitrobenzoic acid) in 1% sodium citrate solution. Read at 412 m μ at once against a reagent blank.

By nitroprusside. Dilute 1 ml. of heparinized blood to 5 ml. and heat to 37°. Precipitate with 2 ml. of 13% sulfosalicylic acid solution. Filter after 10 minutes at 37°. Chill 3 ml. to 10° and add 2 ml. of a solution containing 50 grams of potassium carbonate and 0.086 gram of sodium cyanide per 100 ml. Add 2 ml. of fresh 1% aqueous sodium nitroprusside. Read between 2 and 10 minutes later at 525 m μ . Correct for a blank in which water replaced the nitroprusside solution.

FOLIC ACID, PTEROYLGLUTAMIC ACID, PTEROYL-3-CARBOXY-1-AMINOBUTYRIC ACID

Folic acid is one of the vitamin fractions of the B complex. When it is diazotized (Vol. IV, p. 150) the addition of orthophosphoric acid prevents interference by ferric ion.¹⁶⁹ Thiamine mononitrate interferes.¹⁷⁰ There is no interference by pyridoxine hydrochloride, nicotinamide, inositol, biotin, vitamin B₁₂, *p*-aminobenzoic acid, and calcium pantothenate, strychnine, methionine, choline dihydrogen citrate, glycerol, propylene glycol, ethanol, phenol, ascorbic acid, ferric ammonium citrate,

¹⁶⁸ Leon Lack and Mabel Smith, *Ann. Biochem.* **8**, 217-22 (1964).

¹⁶⁹ E. Haberli, E. Béguin and G. Schenk, *Z. anal. Chem.* **155**, 415-7 (1957).

¹⁷⁰ M. S. Madiwale, S. S. Rao and N. K. Dutta, *Indian J. Pharm.* **24**, 286-9 (1962).

ferrous gluconate, and riboflavin. An alternative is to precipitate iron in ammoniacal solution by hydrogen sulfide, and thereafter evaporate the excess hydrogen sulfide *in vacuo*.¹⁷¹

When a solution of folic acid is boiled with nitric acid and made alkaline with ammonium hydroxide, the color is read at 485 m μ .¹⁷² The fluorimetric method after oxidation is applicable to urine (Vol. IV, pp. 150-1). The sample is defecated with lead acetate before oxidation.¹⁷³ The method is widely applicable.¹⁷⁴

The familiar amine coupling with *N*-(1-naphthyl)ethylenediamine is applicable to determination of folic acid.¹⁷⁵ Riboflavin and ascorbic acid interfere but are removable with acid clays.¹⁷⁶ Folic acid and related compounds are readable by excitation at 360-380 m μ to give their maximum fluorescence at 450-460 m μ .¹⁷⁷ The tetrahydro derivatives and many others are readable by excitation at 300-320 m μ to fluoresce at 360-425 m μ .

Procedure—Multivitamin capsules. Crush a sample equivalent to about 5 mg. of folic acid in 3% dibasic potassium phosphate solution, using not less than 10 ml. for each gram of capsules taken. Heat at 50-60° with swirling until completely dispersed. Cool and dilute to 0.05-0.1 mg. of folic acid per ml. Let settle and filter, or centrifuge a portion to clarify it. Dilute an aliquot with the 3% phosphate solution to a concentration of about 0.005 mg. of folic acid per ml.

To each of 3 tubes, add 2 ml. of prepared sample. To tube 1, add an approximately equivalent amount of standard. Dilute each to 5 ml. with the 3% phosphate solution. To tubes 1 and 2, add 1 ml. of 0.4% potassium permanganate solution. To tube 3, add 1 ml. of water. After 2-3 minutes, add 1 ml. of 2% sodium nitrite solution and 1 ml. of 1:1 hydrochloric acid to each. Mix, and after 2 minutes, add 1 ml. of 5% ammonium sulfamate solution to each. Swirl until the nitrogen dioxide has been released. Add 1 ml. of 0.1% solution of *N*-(1-naphthyl)ethylenediamine. Mix, and after 10 minutes add 1 gram of sodium chloride. Add 10 ml. of isobutyl alcohol and shake vigorously for 2-3 minutes. Centrifuge.

¹⁷¹ S. K. Ganguly and Hrishikesh Bhattacharya, *ibid.* **18**, 361-4 (1956).

¹⁷² Gerardo Verges, *Farmac. nueva* (Madrid) **23**, 419-22 (1958).

¹⁷³ Narimasa Ooi, *Vitamins* (Kyoto) **11**, 46-8 (1956).

¹⁷⁴ N. A. Andreeva, *Biokhimiya* **18**, 571-5 (1953).

¹⁷⁵ U.S. Pharmacopoeia, 16th Ed., pp. 195, 898-9 (1960).

¹⁷⁶ Tsutou Aihara and Kazuo Sato, *Vitamins* (Japan) **9**, 58-60 (1955).

¹⁷⁷ Kosaku Uyeda and J. C. Rabinowitz, *Anal. Biochem.* **6**, 100-108 (1963).

Read the isobutyl alcohol layer against that solvent at 550 $m\mu$ not more than 25 minutes after addition of the color reagent.

Mg. of folic acid = $0.001 C (Au - Ab / As - Au)$

C = concentration in micrograms per ml. in the standard

Au = absorbance of tube 2 (sample)

As = absorbance of tube 1 (standard plus sample)

Ab = absorbance of tube 3 (the blank)

ARGININE, GUANIDINE-1-AMINOVALERIC ACID

The common procedure for determination of arginine is the reaction with hypochlorite or hypobromite and development of the reaction product with α -naphthol or oxine (Vol. IV, pp. 157-8). *N*-Bromosuccinimide or 1,3-dibromo-5,5-dimethyl hydantoin are alternative oxidizing agents.¹⁷⁸ It may also be developed by 2,4-dichloro-1-naphthol.¹⁷⁹ The reaction is applicable in the presence of urea.¹⁸⁰ Metabisulfite or thio-sulfate are alternatives to urea. The developed color fades rapidly and is destroyed by alkali.

Arginine may be determined in the presence of guanidineacetic acid by adsorption on Amberlite CG-50, a weakly cationic resin.¹⁸¹ Each is isolated and determined. The sodium form of the resin is superior to the hydrogen form for this purpose. Nalcite HCR-8, a strongly cationic resin, is used to adsorb interferences from urine samples. The guanidineacetic acid is adsorbed from the arginine-free sample after passage through Amberlite CG-50. Interferences are left in the effluent. From 0.037 micromole to 9.5 micromoles of either component may be measured by this procedure.

Over 13 micromoles of creatinine, 3 micromoles of glutathione, or 5 micromoles of cysteine cause a 50% reduction of color formation by 0.05 micromole of arginine or guanidinoacetic acid. Creatine over 1 micromole produces a greenish yellow color with the reagents. Urea is usually added to prevent color deterioration with hypobromite.¹⁸² Sulfosalicylic acid

¹⁷⁸ G. Ceriotti and L. Spandrio, *Biochem. J.* **66**, 603-7 (1957); Kazuo Satake and J. Murray Luck, *Bull. soc. chim. biol.* **40**, 1743-56 (1958); cf. K. Roszek-Masiak, *Chemia analit.* **9**, 837-42 (1964).

¹⁷⁹ Arline D. Deitch, *J. Histochem. and Cytochem.* **9**, 477-83 (1961).

¹⁸⁰ K. R. Bhattacharya, *Ann. Biochem. Exptl. Med. (Calcutta)* **20**, 57-64 (1960).

¹⁸¹ Chung Wu, *Arch. Biochem. and Biophys.* **85**, 461-70 (1959).

¹⁸² Paolo M. Strocchi and Pierfrancesco Drago, *Ann. chim. (Rome)* **44**, 836-12 (1954).

may replace urea to eliminate excess hypobromite.¹⁸³ Another alternative is urethan.¹⁸⁴

Serum contains interferences that are not precipitated by sulfosalicylic acid. Arginine in serum is therefore determined by precipitation of interferences with trichloroacetic acid. The filtrate should be adjusted to pH 9 before color development. Glycine intensifies the color, possibly by protecting the arginine from oxidative deamination and decarboxylation. Histidine interferes. This reaction is specific for monosubstituted guanidino compounds. Beer's law is followed for 0.00545-0.087 mg. of arginine. The method for urea by diacetylmonoxine (Vol. IV, p. 323) applies to all compounds with a guanidine group and is therefore applicable to arginine.¹⁸⁵

Sample—Urine. Dilute a 24-hour sample to 1500 ml. Develop a 1-2 ml. aliquot with alkaline thymine- α -naphthol.

Plasma. Deproteinize a 1-ml. sample with 2 ml. of 6% trichloroacetic acid solution. Develop a 2-ml. aliquot with alkaline thymine- α -naphthol.

Kidney extract. Homogenize kidney tissue in water and centrifuge to remove debris. Deproteinize the supernatant liquid by heating or by treating with 4% trichloroacetic acid solution. Develop an aliquot equivalent to 10 mg. of tissue, dry weight, with alkaline thymine- α -naphthol.

Procedure—Arginine and guanidineacetic acid by alkaline thymine- α -naphthol. To prepare the α -naphthol reagent, mix equal volumes of 10% sodium hydroxide solution containing 20 mg. of thymine per ml. and 0.04% α -naphthol solution in ethanol. The mixture is stable for 1 day.¹⁸⁶

Convert Amberlite CG-50, H form, to the sodium form by treating with 4% sodium hydroxide solution. Wash with water to remove excess alkali until the pH of the washings is about 10. To prepare sodium hypochlorite solution, dilute Chlorox 1:15.

The apparatus for chromatography may be microfilter-type tube, fritted-disk funnels, or 7.5 \times 100-mm. chromatographic tubes with a

¹⁸³ Shigeru Akamatsu and Takeo Watanabe, *J. Biochemistry* (Tokyo) **49**, 566-9 (1961).

¹⁸⁴ J. P. Salta and Y. Khouvine, *Bull. Soc. chim. biol.* **35**, 697-701 (1953).

¹⁸⁵ R. Rendi, *Experientia* **13**, 21-2 (1957).

¹⁸⁶ John F. Pilsum, R. P. Martin, E. Kito and J. Hess, *J. Biol. Chem.* **222**, 225-236 (1956).

fritted disk and buret-type delivery tip below the stopcock. Add Amberlite CG-50, sodium form to one chromatography tube to a height of 1 cm. To another tube, add Nalcite-HCR-8, without further purification, to a height of 1 cm. Place the Amberlite funnel straight above the Nalcite funnel, with the tip of the former slightly inside the top opening of the latter. A sample may contain 0.05-0.2 micromole each of arginine and guanidineacetic acid. Add the sample dropwise to the Amberlite tube and follow by water. Collect 10 ml. of effluent from the Nalcite tube and discard. Separate the columns and place next to each other. To each, add 4.1% sodium acetate solution slowly until 10 ml. from each tube has been collected. Develop arginine from the Amberlite tube and guanidineacetic acid from the Nalcite tube. If guanidineacetic acid is not present, eliminate the Nalcite tube. If arginine is absent, eliminate elution of the Amberlite column with sodium acetate.

Develop a 4-ml. portion of each effluent. Cool the effluent and all reagents at 2° for a few minutes. To each of the effluents, add 1 ml. of alkaline thymine- α -naphthol solution. After 3 minutes, add 0.5 ml. of sodium hypochlorite solution and mix thoroughly. Read each at 500 m μ after 15 minutes.

By sulfosalicylic acid-oxine-hypobromite. To prepare the hypobromite solution, dissolve 1 gram of bromine in 100 ml. of 5% sodium hydroxide solution. To prepare the sulfosalicylic acid-oxine solution, mix 50 ml. of 5% sulfosalicylic acid solution and 50 ml. of 0.075% glycine solution. Add 0.05 gram of oxine. To a 2-ml. sample, add 2 ml. of sulfosalicylic acid-oxine reagent and filter. Mix 2 ml. of the deproteinized filtrate with 1 ml. of 2.5% sodium hydroxide solution and place in ice water for 15 minutes. Add 1 ml. of hypobromite solution. Dilute within 10 minutes to 10 ml. with water at room temperature. Read at 500 m μ .

α -OXO- δ -AMINOVALERIC ACID

Various imino acids are oxidized to α -oxo- δ -aminovaleric acid. Therefore, the technic that follows is applicable not only to that acid as such, but to *proline*, *ornithine*, and *lysine*.¹⁸⁷ They are then determined by *o*-aminobenzaldehyde. The calibration curve is desirably prepared from the sample plus known amounts of the test substance.

Taking the absorption of proline as 100, the color intensity of other compounds with the reagent follows: hydroxyproline 62.9; ornithine 43;

¹⁸⁷ Lumir Macholan, *Z. physiol. Chem.* **328**, 111-19 (1962).

tryptophan 28.5; lysine 15.7; pipercolic acid 4.3; tyrosine 2.8; histidine and methionine 1.6; glycine 1.4; citrulline and serine 1; arginine, alanine, and phenylalanine 0.6; cysteine, leucine, and threonine 0.4; aspartic acid, glutamic acid, creatine, and valine 0.1.

Procedure— *α -Oxo- δ -aminovaleric acid.* To 1 ml. of sample containing 0.05-0.5 mg. of the test substance, add 3 ml. of 0.1 *M* citrate buffer for pH 5.0 containing toluene as a preservative. Add 1 ml. of a reagent containing 3.6 mg. of *o*-aminobenzaldehyde in water, made fresh daily. Incubate for 60 minutes at 37° and read at 430 m μ against water.

Proline. Ornithine and lysine absent. Prepare a reagent containing 1 volume of 1.25% copper sulfate pentahydrate and 2 volumes of 20% sodium hydroxide. To 1 ml. of sample containing 0.05-0.5 mg. of proline, add 1.5 ml. of the copper reagent and 0.5 ml. of 6% hydrogen peroxide. Mix, and incubate for 5 minutes at 37°. Shake, and incubate for 10 minutes longer at 37°. Chill in ice water. Acidify to pH 5 with 1 ml. of 38% aqueous citric acid. If hydroxyproline is present, heat for 10 minutes at 100°. Pick up the previous method at "Add 1 ml. of a . . ."

Ornithine or lysine present. Adjust 1-3 ml. of sample solution to pH 5. Pass through a 5 \times 100-mm. Zeolite 226 column with the usual technics. The proline and ornithine are retained. Elute the proline with 2, 2, and 2 ml. of water. Recovery can be expected to be 98-99%. Develop the eluate as for the proline samples, which it is not necessary to chromatograph.

Ornithine. After the lysine and proline have passed through, elute the ornithine with 10 ml. of 4% sodium hydroxide solution. Develop as for proline.

ISOLEUCINE, 1-AMINOCAPROIC ACID

Isoleucine is determined in the presence of leucine and valine as methylethyl ketone by reaction with vanillin in an acid medium¹⁸⁸ (Volume IV, pp. 158-60). Beer's law is followed for 0.06-0.24 mg. isoleucine.

Procedure—*Protein hydrolyzates.* To prepare the reagent, dissolve 100 mg. of vanillin in 1 ml. of anhydrous methanol and dilute with concentrated hydrochloric acid to 100 ml.

¹⁸⁸ Barbara Buhlak and Maria Szelegiewicz, *Chem. Anal.* (Warsaw) 6, 83-9 (1961).

Dilute a sample containing 5-20 mg. of isoleucine to 10 ml. with water. Add 5 ml. of 28% sodium nitrite solution. With vigorous stirring, add 2 ml. of 33% sulfuric acid solution dropwise. Let stand for 12 hours at room temperature. Heat at 100° for 15 minutes, cool, and neutralize with 20% sodium hydroxide solution to pH 4-6. Add the solution dropwise, during a 10-minute interval, to 10 ml. of fresh 10% sodium bisulfite solution connected to another flask containing 150 ml. of a boiling solution of 3% potassium permanganate solution in pH 6.8 phosphate buffer, connected in turn to a receiver. Collect 30 ml. of distillate during 30 minutes. To 2 ml., add 2 ml. of reagent and 2 ml. of concentrated sulfuric acid. Let the mixture stand for 30 minutes at room temperature. Add 4 ml. of ethyl acetate, shake, and read at 600 m μ .

LYSINE, 1,5-DIAMINOCAPROIC ACID

When lysine is decarboxylated with bacterial lysine decarboxylase, cadaverine is formed. Cadaverine is then determined with 2,4-dinitrofluorobenzene.¹⁸⁹ Compounds that react with the decarboxylase include diaminopimelic acid, asparagine, ammonium chloride, and ornithine.

The following compounds inhibit the reaction by more than 10%: aminocaprylic acid, proline, histidine, and tyrosine. In plasma samples, the lysine is decarboxylated before deproteinization with zinc sulfate. Beer's law is followed up to 0.16 micromole of cadaverine.

The reaction of the amine group of lysine with 2,4-dinitrofluorobenzene produces a color read at 455 m μ .¹⁹⁰ The absorbance of ninhydrin derivatives α - and ϵ -N-substituted lysines in 2-methoxyethanol permits their determination.¹⁹¹

Lysine in the range of 0.01 to 0.05 mg. is determined by diazotized *p*-nitroaniline.¹⁹² In the presence of histidine and arginine, the sample is passed through an ion-exchange column. A stable red-violet color is formed. After formation of a complex with copper, lysine is determined by potassium thiocyanate and pyridine.¹⁹³

¹⁸⁹ K. J. Carpenter, *Biochem. J.* 77, 604-10 (1960); R. Pion, *Ann. Biol. Animale Biochim. Biophys.* 1, 235-47 (1961); Herbert W. Dickerman and Mary Louise Carter, *Anal. Biochem.* 3, 195-205 (1962).

¹⁹⁰ Susumu Adachi and Takeo Nakanishi, *Nippon Nogei Kagaku Kaishi* 32, 728-32 (1958).

¹⁹¹ Woon Ki Paik and Sand Sanyduk Kim, *Nature* 202, 793-4 (1961).

¹⁹² Juan Claudio Sanahuja and Delia Seone Rios, *Anales bromatol.* 10, 165-73 (1958); *Rev. asoc. bioquim. arg.* 23, 232-7 (1958); *Rev. Farm.* 102, 239-45 (1960).

¹⁹³ M. Bertucat, P. Mesnard and O. Sylla, *Bull. Soc. Pharm. Bordeaux* 101, 37-40 (1962).

Oxidation and phosphomolybdic-phosphotungstic acid reagent (Vol. IV, pp. 161-2) will determine 0.025 mg. of lysine in 2-3 ml. of solution.¹⁹⁴ Hydrolysates are chromatographed at pH 5 on Amberlite IRC 50, acid form. A 0.1 M citrate buffer for pH 5 successively elutes phenylalanine, tyrosine, and lysine.¹⁹⁵ For determination of lysine as α -oxo-S-amino-valeric acid, see page 286.

Sample—*Urine*. Dilute 10 fold or more and develop as cadaverine.

Plasma. Suspend 100 mg. of lysine decarboxylase in 10 ml. of 0.1 M phosphate buffer at pH 7 and centrifuge at 3000 gravities for 15 minutes. Decant the supernatant liquid and resuspend the residue in 10 ml. of water. Add a 0.5-ml. sample to the enzyme suspension. Incubate for 45 minutes at 38°. Add 2 ml. of 10% zinc sulfate solution and 2 ml. of 2% sodium hydroxide solution, and centrifuge for 15 minutes at 3000 gravities. Develop a 1-ml. aliquot as cadaverine, starting at "Add 1 ml. of the supernatant liquid to 0.04 ml. of 0.012% sodium diethyl dithiocarbamate solution . . . ," but use 1 ml. of buffer in place of 0.6 ml.

Procedure—As *cadaverine*. Prepare the lysine decarboxylase as described under "Plasma." To prepare the sodium carbonate buffer, mix 4.2 grams of sodium bicarbonate and 10.6 grams of sodium carbonate, and dilute to 500 ml. with water.

Prepare incubation tubes containing the lysine sample, 1 ml. of 2.7% sodium maleate buffer solution, and 0.1 ml. of 0.02% pyridoxal phosphate solution. Dilute to a final volume of 2.1 ml. Add 0.2 ml. of lysine decarboxylase suspension and incubate for 30 minutes at 38°. Add 0.1 ml. of 1:4 hydrochloric acid and heat at 38° for 10 minutes. Centrifuge at 3000 gravities for 15 minutes.

Add 1 ml. of the supernatant liquid to 0.04 ml. of 0.0125% sodium diethyldithiocarbamate solution. Add 0.1 ml. of 0.14% 1-fluoro-2,4-dinitrobenzene solution and 0.6 ml. of carbonate buffer. Incubate at 65° for 45 minutes. Add 0.4 ml. of 0.8% sodium hydroxide solution in 60% dioxane and incubate at 65° for 1 hour. Let stand at room temperature for 10 minutes. Stir with 5 ml. of chloroform for 60 seconds. Centrifuge

¹⁹⁴ Augustin D. Marenzi and Carlos J. Gómez, *Publ. inst. invest. microquim., Univ. natl.itoral* (Rosario, Arg.) 17, 170-84 (1953); *Anales farm. y. bioquim.* (Buenos Aires) 21, 106-20 (1954); J. P. Zalta and Y. Kouvine, *Bull. soc. chim. biol.* 35, 697-701 (1953).

¹⁹⁵ Naokazu Sakota, Yoshimi Okada and Hiroko Urabe, *Nippon Kagaku Zasshi* 76, 1146-8 (1955).

at 800 gravities for 5 minutes and remove the aqueous layer by aspiration.

Add 2 ml. of the chloroform layer to 2 ml. of 1:10 hydrochloric acid, mix for 60 seconds, and centrifuge at 800 gravities for 2 minutes. Remove the acid layer. Add 2 ml. of 4% sodium hydroxide solution, stir and centrifuge at 800 gravities for 2 minutes. Remove the alkaline layer. Read the washed chloroform layer at 355 $m\mu$ against a reagent blank.

As the copper-lysine complex with potassium thiocyanate and pyridine. Adjust the pH of a 20-ml. sample to 8 and shake with a suspension of cupric hydroxide. Filter. Precipitate 10 ml. of filtrate by addition of a 1% solution of *p*-nitrobenzaldehyde in ethanol. Adjust the pH to 9.6, filter, and wash with water. Dissolve the precipitate in about 15 ml. of 1:100 hydrochloric acid and dilute to 25 ml. with water. To 5 ml. of this solution, add 5 ml. of 2% solution of potassium thiocyanate, 2 ml. of pyridine, and 5 ml. of chloroform. Separate the chloroform layer and read at 720 $m\mu$.

By diazotized p-nitroaniline. To prepare the reagent, dissolve 1.5 grams of *p*-nitroaniline in 40 ml. of concentrated hydrochloric acid. Add 40 ml. of water and mix. To a 25-ml. aliquot, add 0.75 ml. of 10% sodium nitrite solution.

Dilute a 1-ml. sample containing 0.03-0.3 mg. of lysine to 5 ml. with water and add 1 ml. of freshly prepared reagent. After 5 minutes, add 3 ml. of 16% sodium carbonate solution. Dilute after 20 minutes to 15 ml. with a solution containing 40 grams of sodium hydroxide, 100 ml. of 95% ethanol, and 140 ml. of water. Mix, and read at 520 $m\mu$ against a reagent blank.

In the presence of arginine and histidine. To prepare the Amberlite IRC 50 resin, wash consecutively with sodium hydroxide solution, pH 7 buffer, and water. Pass a mixture of lysine, arginine, and histidine through 2 grams of Amberlite IRC 50 in a 3×100 -mm. column at a flow rate of 0.3 ml. per minute. Histidine passes through the column. To elute arginine and lysine, use 1:10 hydrochloric acid and water. Adjust a 10-ml. aliquot to pH 4 with 4% sodium hydroxide solution and pass through a column of Amberlite IRA 400, which has been previously washed with 4% sodium hydroxide solution and water, at a rate of 0.3 ml. per minute. Elute with 40 ml. of a pH 4.5 acetate buffer.

To 5 ml. of the eluate, add 1 ml. of diazotized *p*-nitroaniline and let stand for 5 minutes. Add 3 ml. of 22.5% sodium carbonate solution and mix every 5 minutes for 40 minutes. Dilute to 15 ml. with a solution con-

taining 40 grams of sodium hydroxide, 100 ml. of 95% ethanol, and 140 ml. of water. Read at 520 $m\mu$ against a reagent blank.

By dinitrofluorobenzene. Milk. Add a 0.5-ml. sample to 100 mg. of sodium bicarbonate. Shake with 2 ml. of 2.5% 2,4-dinitrofluorobenzene solution in 95% ethanol for 2 hours in the dark. Evaporate the ethanol at 100°. Add 6 ml. of 1:1 hydrochloric acid, seal the tube, and heat at 105° for 15 hours. Filter, dilute to 10 ml., and extract a 2-ml. aliquot with three 10-ml. portions of ether shaking 1 minute for each extraction. Dilute 1 ml. of the aqueous layer to 10 ml. and adjust the pH to 4.5 with 1:20 acetic acid or 4% sodium hydroxide solution. Read at 455 $m\mu$ against a blank containing all the reagents except dinitrofluorobenzene.

By ninhydrin. α - and ϵ -substituted derivatives. Mix 0.1 ml. of sample containing a micromole of the isomers. Add 0.9 ml. of a 4% solution of ninhydrin in 2-methoxyethanol. Mix, and heat at 100° for exactly 10 minutes. Chill in ice water for 1 minute and add 7 ml. of ice water. Mix, and read at 516 $m\mu$ and 570 $m\mu$.

AVAILABLE LYSINE

In a protein, only that part of the lysine is available which has the epsilon amino groups free. After dinitrophenylation, the derivative of the available lysine is isolated by column chromatography and read in hydrochloric acid-methyl ethyl ketone solution.¹⁹⁶ *O*-Dinitrophenylated tyrosine is also present but does not absorb at 435 $m\mu$.

Procedure—*By nitrophenylation.* Prepare a chromatographic column from 10-mm. \times 16-cm. tubing having a medium porosity sintered glass disc and Teflon stopcock. Fuse a 125-ml. bulb to the top of the column. This has an 18/7 ball-joint at the top of the bulb to facilitate operation under pressure.

Pour a slurry of Amberlite IR-120, size B, in the sodium form, into the column to build up a column of about 6 cm. After settling about 1 cm. by gravity, compact with slight air pressure. Wash with 1:3 hydrochloric acid until acid comes through and adjust to about 5.5 cm. Keep moist at all times. After use, regenerate by washing successively with water, 4% sodium hydroxide solution, water, and 1:3 hydrochloric acid.

¹⁹⁶ S. Raghavendar Rao, Fairie Lyn Carter and Vernon L. Frampton, *Anal. Chem.* 35, 1927-30 (1963); A. Rutkowski, J. Budny and B. Chodkowska-Lossow, *Chem. Anal., Warsaw* 9, 255-60 (1964).

Meal. Grind a defatted sample to pass a 40-mesh sieve. Weigh out 5 grams, which will contain 30-50 mg. of nitrogen, into a 1-liter round-bottom flask. Add 10 ml. of 10% aqueous sodium bicarbonate and mix by swirling. After 10 minutes, add 0.3 ml. of 2,4-dinitrofluorobenzene in 10 ml. of absolute ethanol. Again, mix by swirling, and wash down the sides of the flask with 3 ml. of ethanol. Shake for 2 hours in subdued light with a wrist-motion shaker. Reaction of the free epsilon amino groups and the 2,4-dinitrofluorobenzene is now complete. Evaporate the alcohol and most of the water by an air stream. Extract with four successive 50-ml. portions of peroxide-free diethyl ether. In each case, decant the ether from the residue in the flask. Dry the residue in the flask by an air stream.

Add 200 ml. of concentrated hydrochloric acid and reflux overnight. Cool, and filter through sintered glass. Wash the filter and dilute the filtrate and washings to 250 ml.

Transfer an aliquot of 2-4 ml. of hydrolysate containing 0.1-0.3 mg. of epsilon-dinitrophenyllysine to the column without disturbing the surface of the resin. Drive this into the column by air pressure until the meniscus coincides with the surface of the column packing. Wash down the sides of the reservoir with three successive 1-ml. portions of 1:3 hydrochloric acid and drive each into the column under pressure. Develop the column with 37 ml. of additional 1:3 hydrochloric acid. Flow should be at 12-15 drops per minute. The hydrochloric acid elutes dinitrophenol and other yellow components.

The eluting solvent is a fresh mixture of 3 volumes of 1:3 hydrochloric acid with 1 volume of methyl ethyl ketone. Elute with 45 ml. of this. The progress of the lysine derivative down the column is visible. The first 12-13 ml. of this solvent are discarded, the lysine derivative then being about 0.5 cm. from the bottom of the column. Filter the next 22 ml. of effluent and wash the filter with solvent to dilute to 25 ml. Read at 435 $m\mu$ against the solvent mixture.

SARCOLYSINE, 3[*p*-DI-(2-CHLOROETHYL)AMINOPHENYL]ALANINE

Sarcolysine in methanol is read in the ultraviolet.¹⁹⁷

Procedure—Tablets. Powder 10 tablets and take a sample corresponding to 50 mg. of sarcolysine. Shake with 100 ml. of methanol for

¹⁹⁷ J. Blazek and J. Kraemer, *Ceskosl. Farm.* **11**, 210-12 (1962).

15 minutes. Dilute to 250 ml. and let stand to settle. Dilute 5 ml. of the clear upper layer to 100 ml. with methanol and read at 262 m μ .

KYNURENINE, γ -AMINO- α -(*o*-AMINOPHENYL)GLUTACONIC ACID

Kynurenine in the range of 0.002-0.018 mg. per ml. is developed by formation of a diazo dye with ethyl-1-naphthylamine¹⁹⁸ (cf. Vol. IV, p. 164). Tryptophan and anthranilic acid give the same color with the reagent, but the color with tryptophan can be extracted with ether, and that from anthranilic acid, with ethyl acetate.

The products of tryptophan metabolism, which include kynurenine, 3-hydroxykynurenine, *N*- α -acetylkynurenine, anthranilic acid, 3-hydroxyanthranilic acid, kynurenic acid, xanthurenic acid, and the 8-methyl ether of xanthurenic acid, are separated by paper chromatography by a mixture of *n*-butanol, acetic acid and water, and by water.¹⁹⁹ Then each is determined.

Kynurenic acid, *N*- α -acetylkynurenine, and xanthurenic acid 8-methyl ether are eluted with ethanol and read in the ultraviolet. Kynurenine and anthranilic acid are developed with *p*-dimethylaminobenzaldehyde. 3-Hydroxykynurenine, xanthurenic, and 3-hydroxyanthranilic acids are eluted with water and developed with a diazo reagent.

If both xanthurenic acid and its methyl ether are present, two chromatograms must be prepared, since the spots overlap. On the first chromatogram, develop xanthurenic acid by diazotization after elution. On the second chromatogram, elute the 8-methyl ether with ethanol and read at 237 m μ . Xanthurenic acid does not interfere. Beer's law is followed for *N*- α -acetylkynurenine and xanthurenic acid 8-methyl ether for 0.001-0.015 mg. The procedure is applicable for 0.001-0.005 mg. of the other components.

In urine, an alternative is to chromatograph on the acid form of an ion-exchange resin, elute with strong acid, and develop the fluorescence with cyanogen bromide.²⁰⁰

Sample—Urine. Prepare a chromatography paper 57 \times 46 cm. Apply a 0.1-ml. sample near the edge of the chromatography paper. Develop by chromatography.

¹⁹⁸ Takeyo Nishikawa and Kiyomasa Kometani, *Osaka Shiritsu Daigaku Igaku Zasshi* **9**, 4997-5000 (1960).

¹⁹⁹ D. Coppani, C. A. Benassi and M. Montorsi, *Clin. Chem.* **5**, 391-401 (1959).

²⁰⁰ Yukito Kotake, Minoru Tsuji and Norikiko Hasagana, *Proc. Japan Acad.* **37**, 48-53 (1961).

Blood serum. Add a 0.5-ml. sample, in droplets, to 5 ml. of 1:1 absolute ethanol and anhydrous acetone mixture with continuous stirring. Centrifuge, and decant the supernatant liquid. Wash the precipitate with 2-ml. portions of the ethanol-acetone mixture and add the washings to the supernatant liquid until the total volume of liquid is 10 ml. Chromatograph 0.1 ml. directly, or concentrate if the content is low.

Spinal fluid. Gently heat 1 ml. of sample to boiling. Add 1 drop of 10% trichloroacetic acid solution and heat at 50° for 5 minutes. Cool and centrifuge. Spot 0.1 ml. on the chromatography paper.

Procedure—By chromatography. The samples are spotted at a distance of 8 cm. from the edge of the paper. Dry with warm air. Put the sheets in chromatography cabinets for at least 4 hours before adding the solvent mixture. Chromatograph with a 4:1:5 mixture of *n*-butanol, acetic acid, and water, and allow the compounds to separate for 14 hours at 20°. Dry at 40°. For the second run, use water, and allow 4 hours at 20°. Dry at 40° and observe the spots at 365 m μ . See Table 17 for details.

TABLE 17. CHROMATOGRAPHIC BEHAVIOR OF
SOME COMPOUNDS

<i>Substances</i>	<i>Mean R_F</i>	
	<i>butanol-acetic acid—H₂O</i>	<i>Mean R_F H₂O</i>
Kynurenine	0.37	0.67
3-Hydroxykynurenine	0.30	0.56
<i>N</i> α -Acetylkynurenine	0.82	0.81
Anthranilic acid	0.88	0.66
3-Hydroxyanthranilic acid	0.85	0.60
Kynurenic acid	0.56	0.57
Xanthurenic acid	0.56	0.45
Xanthurenic acid 8-methyl ether	0.55	0.51

To prepare the diazotized sulfanilic acid, mix equal volumes of 0.5% sulfanilic acid solution in 2% hydrochloric acid and 0.5% sodium nitrite solution. Place pieces of paper with 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and xanthurenic acid in flasks. Add 3.8 ml. of water to each. After 15-16 hours, and at 15°, add 1 ml. of diazotized sulfanilic

acid reagent and 0.2 ml. of pyridine. Read xanthurenic acid immediately at 15° at 510 $m\mu$. Read the remaining compounds at 450 $m\mu$ after 60-80 minutes.

Elute the cuts of kynurenic acid with 5 ml. of 95% ethanol and read at 243 $m\mu$. *N*- α -Acetylkynurenine gives a blue fluorescence. Elute with 95% ethanol and read at 227 $m\mu$. Xanthurenic acid 8-methyl ether gives a brilliant blue fluorescence. Elute with ethanol and read at 237 $m\mu$. Alternatively, elute cuts corresponding to kynurenine and anthranilic acid with 5 ml. of 1% *p*-dimethylaminobenzaldehyde solution in 50% acetic acid. Read at 450 $m\mu$. The calculated analytical factors permit the conversion of the extinction observed to the concentration of compound present in the spot.

Calculated analytical factors ($A = 1/E$), E = mg. per ml.:

Kynurenine	124.9
3-Hydroxykynurenine	65.3
Anthranilic acid	24.7
3-Hydroxyanthranilic acid	96.6
Xanthurenic acid	37.6
Kynurenic acid	33.1
<i>N</i> - α -Acetylkynurenine	92.60
Xanthurenic acid 8-methyl ether	45.73

By ethyl-1-naphthylamine-hydrobromide. To a 5-ml. ice-cold sample, add 0.3 ml. of 1:59 hydrochloric acid and 0.2 ml. of 0.14% sodium nitrite solution. Let stand for 30 minutes in ice. Add 1 ml. of 0.3% ethyl 1-naphthylamine-hydrobromide solution in 90% ethanol. After 1 hour at room temperature, read at 550 $m\mu$.

By cyanogen bromide. Urine. To 1-2% of a 24-hour specimen, add 5 ml. of 1:11 hydrochloric acid and dilute to 50 ml. Add to a 1 \times 7-cm. column of Dowex 50-X-12 in the hydrogen form. Wash successively with 50 ml. of 1:110 hydrochloric acid, 100 ml. of 1:21 hydrochloric acid, 100 ml. of 1:11 hydrochloric acid, and 100 ml. of 1:5 hydrochloric acid. Elute kynurenine from the column with 100 ml. of 1:2.5 hydrochloric acid. Neutralize 2 ml. of eluate to pH 7 with 32% sodium hydroxide. Dilute to 6 ml. with a phosphate buffer for pH 7. Add 2 ml. of 4% cyanogen bromide solution and heat for 8 minutes at 70-80°. Cool in ice water and add 2 ml. of 8% sodium hydroxide solution. Read the fluorescence at 440 $m\mu$.

3-HYDROXYKYNURENINE

This compound is oxidized and then rearranged for reading.²⁰¹

Procedure—Place 0.5 ml. of sample in a centrifuge tube. Add 0.4 ml. of 0.6 M phosphate buffer for pH 6.8. Chill to 0° and add 0.1 ml. of 3.3% potassium ferricyanide to oxidize to ommochrome xanthommatin. Add 10-20 mg. of ascorbic acid to convert to red dihydroxanthommatin. Add 0.1 ml. of concentrated hydrochloric acid and extract the color body with 1.2 ml. of butanol. Read at 492 m μ .

PROLINE, PYRROLIDENE-2-CARBOXYLIC ACID

Modifications of the method of determination of proline by ninhydrin have been developed (see Vol. IV, p. 165). If up to 2 micromoles of glycine, alanine, valine, leucine, isoleucine, serine, threonine, arginine, asparagine, or glutamic acid is present per ml. of proline solution, the heating is decreased to 30 minutes and the ninhydrin concentration is reduced to 20 mg. per ml. None of the 10 amino acids interferes with the proline when present in a concentration of 1-2 micromoles per ml. Interference from methionine, phenylalanine, tyrosine, glutamine, hydroxyproline, histidine, and tryptophan is lessened by these modifications.²⁰²

If the heating time is shortened, interferences from dipeptides and tripeptides, which hydrolyze to amino acids, is lessened. The following do not interfere: leucinamide, hippuric acid, and ammonium sulfate. Even with these modifications, proline cannot be estimated in solutions containing more than 0.1-0.5 micromole of glutamine, tryptophan, hydroxyproline, tyrosine, and histidine. More than 2 micromoles of the remaining amino acids interfere. For determination by ninhydrin in serum, the removal of protein by trichloroacetic acid or tungstic acid gives high results. This can be corrected by precipitating with a mixture of 95 parts of absolute ethanol and 1 part of 1:100 acetic acid.²⁰³

In the standard method, 1.8 micromoles of glycine is added to the proline solution. Interferences of lysine, hydroxylysine and ornithine with the determination of proline with ninhydrin are removed by shaking with

²⁰¹ B. Linzen, *Z. physiol. Chem.* **333**, 145-8 (1963).

²⁰² Michael Messer, *Anal. Biochem.* **2**, 353-9 (1961).

²⁰³ George K. Summer and Jean A. Hawes, *Proc. Soc. Exptl. Biol. Med.* **112**, 402-4 (1963).

Permutit.²⁰⁴ Proline may be determined in the presence of pipercolic acid by adding hydrochloric or sulfuric acids to inhibit its color formation with ninhydrin.²⁰⁵ The development of the color depends on the amount of reagent, optimal amount for pipercolic acid and proline being 100 times the imino acid concentration. The sample is passed through Amberlite IR-4B to remove interference from leucine. Passage through Amberlite IRC-50 removes arginine and ornithine, which is converted to arginine by nitrous acid. Citrulline interferes. Proline developed by ninhydrin in protein hydrolysates is extractable with isobutanol for reading.²⁰⁶ Any residual hydrochloric acid radically reduces the color. For proline as determined by pyrocatechol see dibutylamine, page 137, but read after 2 hours.

An isatin method is made specific for proline by treating the chromatogram of the amino acids with dilute acid. Interference from amino acids, including hydroxyproline, purines, pyrimidines, vitamins, and accessory growth factors are eliminated. Beer's law is followed for 0.001-0.016 mg. proline. The color is read at 600 m μ .²⁰⁷

Proline is determined by *o*-aminobenzaldehyde by a technic described under α -oxo- δ -aminovaleric acid, page 286. For simultaneous determination of proline and pipercolic acid, see page 298.

Sample—Urine. Reflux urine samples with phosphoric acid containing 192 grams per liter for 24 hours to liberate proline from conjugates or peptides. Finally, dilute the urine samples 20-fold with the phosphoric acid. Develop with ninhydrin.

Plasma. Dilute deproteinized samples 10-fold and develop with ninhydrin.

Protein hydrolyzates. To prepare the ion exchange resin, wash Amberlite IR-4B alternately and twice with 1:5 hydrochloric acid and 8% sodium hydroxide solution. Continue the last sodium hydroxide wash until the resin is chloride-free. Wash the resin with water until the pH falls below 8. Add the prepared Amberlite IR-4B to a 0.9 \times 10-cm. column and wash with water until neutral. The volume of the packed resin should be 0.9 \times 6 cm.

²⁰⁴ Walter Troll and John Lindsley, *J. Biol. Chem.* **215**, 655-60 (1955).

²⁰⁵ Richard S. Schweet, *ibid.* **208**, 603-13 (1954).

²⁰⁶ M. A. Torban and V. I. Smyslyvaeva, *Zhur. Anal. Khim.* **16**, 645-6 (1961).

²⁰⁷ Arthur E. Pasicka and Joseph F. Morgan, *Proc. Soc. Exptl. Biol. Med.* **93**, 54-7 (1956).

Hydrolyze a 50-mg. protein sample in a sealed tube with 4 ml. of 1:1 hydrochloric acid for 18 hours at 105°. Remove insoluble humin by centrifuging and wash with 1 ml. of water. Mix 0.3 ml. of 20.7% sodium nitrite solution with 0.7 ml. of 10:4 hydrochloric acid. When the reaction stops, add to 1 ml. of the protein hydrolyzate and heat with shaking for 2.5 minutes at 122°. Cool immediately. To remove strong anions, add the sample to the column and adjust the flow rate to 0.1 ml. per minute. Wash the column with three 2-ml. portions of water. Collect the eluate and washings, and dilute to 10 ml. The final pH should be above 4. The optimum range for proline determination is 0.003-0.008 mg. per tube. Therefore, dilute accordingly with glacial acetic acid. If the proline level is low, concentrate the solution in a vacuum desiccator. Develop with ninhydrin.

Procedure—*By ninhydrin.* As a reagent, dissolve 125 mg. of ninhydrin in 3 ml. of glacial acetic acid and 2 ml. of 59% phosphoric acid by heating to 70°. Shake a solution containing 1-6 mg. of proline per liter with 0.1 the weight of Permutit for 5 minutes. Heat 5 ml. of the resulting solution with 5 ml. of glacial acetic acid and 5 ml. of ninhydrin reagent at 100° for 1 hour in covered tubes. Cool to room temperature and extract with 5 ml. of benzene by shaking vigorously for 5 minutes. Read the benzene phase at 515 m μ .

Simultaneously with pipecolic acid. To prepare the ninhydrin reagent, dissolve 372 mg. of ninhydrin in 20 ml. of glacial acetic acid. Adjust the water volume of an aliquot containing 0.0015-0.01 mg. of proline to exactly 0.05 ml. Add 0.2 ml. of a 1:99 hydrochloric acid-glacial acetic acid mixture. Dilute with glacial acetic acid to 3.85 ml. Add 0.3 ml. of ninhydrin reagent. Cover with an aluminum cap and heat in a glycerol bath at 121-122° for exactly 5 minutes. Cool in ice immediately to room temperature. Adjust the solution to 4.15 ml. with glacial acetic acid and read at 530 and 560 m μ within 15 minutes.

Calculate the contribution of proline to color at 530 m μ as follows:

$$DP_{530} = 1.53 D_{530} - 0.93 D_{560}$$

where D_{530} and D_{560} are the observed optical densities at the wave length indicated, and DP_{530} is the optical density of proline at 530 m μ .

By paper chromatography with isatin. Dry a 5-ml. sample *in vacuo* over sulfuric acid and reconstitute with 0.2 ml. of deionized water. Use

a 0.01-ml. portion as a sample. Develop one-dimensional descending paper chromatograms for 18 hours with a solvent system of either *n*-butanol, acetic acid, and water, or *n*-butanol, ethanol, and water. Dry at 110° for 2-3 minutes. Dip in 0.4% isatin solution in *n*-butanol that contains 4% of acetic acid. Reheat at 110° for 10-15 minutes.

Treat the chromatogram with 1:110 hydrochloric acid and dry at room temperature for 10-15 minutes to intensify the blue proline color and allow for fading of other colored spots. Wash the spots with water while the chromatogram is still damp, to remove excess isatin. Cut appropriate areas from the chromatograms and fix to the inner walls of 1-cm. Corex cells filled with water. Read at 600 m μ against a cell containing untreated filter paper.

HYDROXYPROLINE, 4-HYDROXYPYROLIDENE-2-CARBOXYLIC ACID

A common procedure for hydroxyproline involves its oxidation with hydrogen peroxide to form Δ^1 -pyrroline-4-hydroxycarboxylic acid (Vol. IV, p. 167). This product forms a red color with *p*-dimethylaminobenzaldehyde. To prevent the decomposition of this red chromogen when heated in acid, *n*-propanol is added to the reagent.²⁰⁸ Optimal results are obtained with 45.5% of *n*-propanol in the reaction mixture. Color development and destruction are accelerated by temperature.²⁰⁹ Correction for interferences may be made by reading at 500 m μ and 560 m μ , or by extraction of the interferences with chloroform. Tyrosine is adsorbed on Norite to eliminate interferences in liver samples.²¹⁰ Hydrogen peroxide may be replaced by sodium peroxide as the oxidizing agent.²¹¹ Beer's law is followed up to 0.02 mg. of hydroxyproline.

A modification of the reaction of hydroxyproline with *p*-dimethylaminobenzaldehyde lowers the concentration of hydrogen peroxide. This produces a more intense color and reduces interference from an equal amount of tyrosine to less than 1%.²¹² It is applicable in the range of 0.25-50 micrograms to microtome sections. Hydroxyproline in collagen and gelatin is determined by the *p*-dimethylaminobenzaldehyde reagent in the presence of sulfuric acid.²¹³

²⁰⁸ Ferenc Hutterer and Edward J. Singer, *Anal. Chem.* **32**, 556-8 (1960).

²⁰⁹ Jeanette Blomfield and J. F. Fahrar, *ibid.* **36**, 950-2 (1964).

²¹⁰ I. Gordon Fels, *Clin. Chem.* **4**, 62-5 (1958).

²¹¹ Isaac J. Bekhor and Lucien A. Bavetta, *Anal. Chem.* **33**, 1807 (1961).

²¹² B. W. Grunbaum and D. Gluck, *Arch. Biochem. and Biophys.* **65**, 260-7 (1956).

²¹³ D. S. Miyada and A. L. Tappel, *Anal. Chem.* **28**, 909-910 (1956).

Beer's law is followed within the range of 0.001-0.015 mg. of hydroxyproline. At 560 $m\mu$, there is a 2% and a 1.3% interference with hydroxyproline from tyrosine and tryptophan at equimolar concentrations. Hydroxyproline is oxidized in alkaline solution by cupric ion and hydrogen peroxide to Δ^1 -pyrroline-4-carboxylic acid. On acidification, this becomes pyrrole-2-carboxylic acid.²¹⁴ Extraction of this with ethyl ether avoids interference by colored oxidation products of tryptophan and tyrosine. Thereafter, it is developed with *p*-dimethylaminobenzaldehyde.

Another oxidizing agent for hydroxyproline is chloramine-T, 1,4-chlorazone.²¹⁵ In this method, hydroxyproline is oxidized in the presence of alanine as a stabilizer. Any amino acids or similar substances present in urine or tissue hydrolyzates do not influence the yield of pyrrole. The pyrrole is extracted into toluene at pH 8-8.5. The oxidation products Δ^1 -pyrroline-4-hydroxy-2-carboxylic acid and pyrrole-2-carboxylic acid do not interfere. This will determine 0.005 mg. of hydroxyproline in the presence of over 50 mg. of other amino acids.

Hydroxyproline is determined by acid hydrolysis in the presence of stannous chloride, which prevents formation of humin.²¹⁶ A correction is applied for the interference of tyrosine and the loss of hydroxyproline during hydrolysis. This procedure is superior to alkaline hydrolysis, in which both tyrosine and tryptophan interfere.

Procedure—By *p*-dimethylaminobenzaldehyde. Oxidation by cupric ion and hydrogen peroxide. General. To 1 ml. of sample, add 1 ml. of 0.25% solution of cupric sulfate pentahydrate and mix. Add 1 ml. of 10% sodium hydroxide solution and mix. Add 1 ml. of 6% hydrogen peroxide and shake vigorously for 5 minutes. Add 0.1 ml. of 1.4% solution of ferrous sulfate heptahydrate. Shake for 6 minutes and add 4 ml. of 1:9 sulfuric acid. Extract with 10 ml. and 5 ml. of ethyl ether. Combine the ether extracts and extract the pyrrole-2-carboxylic acid from 10 ml. with 3 ml. of 0.4% sodium hydroxide solution. Acidify 2 ml. of the alkaline extract with 3 ml. of 1:9 sulfuric acid. Add 1 ml. of a 5% solution of *p*-

²¹⁴ I. I. Pil'v and A. A. Titaev, *Lab. Delo* 9, 22-5 (1963); J. Bromfield and J. F. Farrar, *Anal. Chem.* 36, 950-2 (1964).

²¹⁵ H. Stegemann, *Z. physiol. Chem.* 311, 41 (1958); Darwin J. Prockop and Sidney Udenfriend, *Anal. Biochem.* 1, 228-39 (1960); Imanuel Bergman and Roy Loxley, *Anal. Chem.* 35, 1961-5 (1963); O. Dahl and K.-A. Persson, *Acta Chem. Scand.* 17, 2499-2503 (1963).

²¹⁶ Eugen Wierbicki and F. E. Deatherage, *J. Agr. and Food Chem.* 2, 878-82 (1954).

dimethylaminobenzaldehyde in propanol. Heat at 70-76° for 16 minutes and cool. Read at 540 $m\mu$ against a blank of 2 ml. of 0.4% sodium hydroxide solution, 3 ml. of 1:9 sulfuric acid, and 1 ml. of aldehyde reagent.

Liver. To prepare the Norite, boil 50 grams with 1.5 liters of 5% acetic acid solution for 10 minutes. Filter with suction and wash with 1 liter of water. Add 100 ml. of 1% sodium cyanide solution to the funnel with stirring. Wash with 2 liters of water and air dry.

Homogenize a sample with 100 ml. of a 1:1 acetone-ether mixture. Let stand overnight at room temperature. Air-dry to a powder by suction and weigh. Add 20 ml. of 1:1 hydrochloric acid to the sample, seal, and hydrolyze at 200° for 20 hours. Cool, break the seal, and filter through sintered glass. Neutralize a 10-ml. portion of the filtrate to Congo red with 12% sodium hydroxide solution. Shake 10 ml. of the neutralized portion for 15-minute periods with two 1-gram portions of Norite. Filter and wash the Norite with three 10-ml. portions of 1:20 acetic acid, adding the washings to the filtrate. Dilute to 50 ml. with 1:20 acetic acid.

To prepare *p*-dimethylaminobenzaldehyde reagent, dissolve 100 grams in 200 ml. of 95% ethanol by heating at 70°. Add charcoal and heat and stir for 5 minutes. Filter with suction. Precipitate *p*-dimethylaminobenzaldehyde with an excess of water. Wash the precipitate on a Büchner funnel with water and dry *in vacuo*. Repeat until a white product that gives a colorless solution in propanol is obtained. As a reagent, prepare a 5% solution in *n*-propanol. To a 1-ml. sample containing 0.001-0.015 mg. of hydroxyproline in 1:20 acetic acid, add 1 ml. of 0.16% cupric sulfate pentahydrate solution, 1 ml. of 8% sodium hydroxide solution, and 1 ml. of 6% hydrogen peroxide. Shake for 5 minutes and heat at 80° for 5 minutes with shaking. Cool in ice. For samples of low hydroxyproline content, use 0.8% cupric sulfate solution.

Add 4 ml. of 1:23 sulfuric acid with shaking and 2 ml. of 5% *p*-dimethylaminobenzaldehyde solution in 1-propanol. Shake for 3 minutes and develop the color at 80° for 30 minutes. Read at 560 $m\mu$ against a reagent blank.

Urine and other complex samples. A suitable urine sample is 0.1-1 ml. A protein solution should contain 0.2-4 mg. of protein per ml. Dilute the sample to 2 ml. with water or with an internal standard, and add 2 ml. of concentrated hydrochloric acid. Seal and autoclave urine or other peptide solutions at 124° for 3 hours, tissue homogenates or proteins for

15-24 hours. If more than a 1-ml. sample of urine or more than a 5-mg. sample of protein is desired, use a larger volume of acid and concentrate in a rotary vacuum evaporator after hydrolysis.

To prepare the humin precipitant, mix 20 grams of 200-400 mesh cation-exchange resin, AG 1-X8 chloride form, with 10 grams of Norit A. Wash several times with 1:1 hydrochloric acid and dry to a fine powder with ethanol and ether.

Dilute a portion of the hydrolyzed sample containing 0.1-1.0 micro-mole of hydroxyproline to 8 ml. The final hydrochloric acid concentration should be 2 to 6 *N*. Add up to 1 ml. of the resin-charcoal mixture, stir, and centrifuge at 5000 gravities for 10 minutes. To 2-4 ml. of the supernatant liquid, add 1 drop of 1% phenolphthalein solution. Adjust the pH to a faint pink color with 50% potassium hydroxide solution, finishing with 0.56% potassium hydroxide solution. Cool to room temperature.

To prepare the potassium borate solution, mix 61.84 grams of boric acid and 225 grams of potassium chloride in 800 ml. of water, and adjust the pH to 8.7 with 50% potassium hydroxide solution. Dilute to 1 liter. For the alanine solution, dissolve 10 grams in 90 ml. of water, adjust the pH to 8.7 with 50% potassium hydroxide solution and dilute to 100 ml. To prepare *p*-dimethylaminobenzaldehyde reagent, slowly add 27.4 ml. of concentrated sulfuric acid to 200 ml. of absolute ethanol and cool. Add 120 grams of *p*-dimethylaminobenzaldehyde to 200 ml. of absolute ethanol and slowly stir the acid-ethanol into this mixture.

Dilute the sample to 8 ml. and saturate with an excess of potassium chloride. Readjust the pH if necessary. Add 2 ml. of borate buffer at pH 8.7 and 1 ml. of alanine solution. Oxidize at room temperature with 2 ml. of 5% chloramine-T solution in methyl Cellosolve. After 20 minutes, stop the oxidation by addition of 6 ml. of 3.6% sodium thiosulfate solution. Again, saturate the solution with potassium chloride and shake vigorously 100 times with 10 ml. of toluene. Centrifuge briefly at a low speed. If an emulsion remains, agitate gently and recentrifuge. Discard the toluene. If large amounts of interferences are suspected, repeat the extraction with toluene. Stopper, and heat at 100° for 30 minutes to form the pyrrole. Cool under running water and shake vigorously with 10 ml. of toluene 100 times. Centrifuge to separate the phases. To 5 ml. of the clear toluene phase, add 2 ml. of *p*-dimethylaminobenzaldehyde reagent and stir rapidly. Read at 560 $m\mu$ after 15 minutes.

To measure free hydroxyproline in urine and other complex samples, place up to 4 ml. of the unhydrolyzed sample in a large culture tube, adjust the pH, and oxidize the sample as above.

Beef muscle. Prepare a 1:1 water slurry of 8 grams of sample. Reflux with 3 ml. of water, 10 ml. of concentrated hydrochloric acid, and 0.7 gram of stannous chloride for 7 hours. Neutralize with 24% sodium hydroxide solution and adjust the pH to 8 with saturated sodium carbonate solution to prevent formation of stannite ions and to facilitate removal of stannous hydroxide. Cool in ice-water and dilute to 100 ml. with water. After 30 minutes, filter with suction to remove the stannous hydroxide precipitate. Filter the hydrolysate and develop as for liver, starting at "To a 1-ml. sample . . ." Correct for tyrosine. The actual amount of tyrosine present in the hydrolyzate is 0.928% on wet basis. Based on a 20 microgram sample of hydroxyproline in a 40-mg. muscle sample, the following is the correction for tyrosine:

$$\frac{0.300}{20} \times \frac{1.3}{100} \times \frac{0.928}{100} \times 40,000 = 0.072 \text{ absorbance unit}$$

where 0.300 is the sample reading and 1.3 is the correction factor for the yellow color of the hydrolyzate per 40 mg. of sample.

Collagen and gelatin. To a 1-ml. sample containing 0.001-0.015 mg. of hydroxyproline, add 1 ml. of 0.16% cupric sulfate solution, 1 ml. of 8% sodium hydroxide solution, and 1 ml. of 6% hydrogen peroxide solution. Shake on a rotator for 5 minutes and then in an 80° bath for 5 minutes. Cool in ice water. Add 4 ml. of 1:23 sulfuric acid with shaking. Add 2 ml. of 5% *p*-dimethylaminobenzaldehyde solution in 1-propanol and shake for 3 minutes. Heat at 80° for 30 minutes. Cool and read at 560 mμ against a reagent blank.

Microtome sections. Cut Pyrex glass tubes 50 mm. long and 5 mm. in inside diameter, containing a volume of 0.5 ml. Freeze-dry individual microtome sections and weigh. Drop each section into a 0.5 ml. tube, add 20 microliters of 1:1 hydrochloric acid and seal. Heat in an oven at 170° for 1 hour and centrifuge for 1-2 minutes. Cut open, and dry in a vacuum desiccator over sodium hydroxide for 20 minutes.

Add 10 microliters each of 0.16% cupric sulfate solution, 10% sodium hydroxide solution, and 2% hydrogen peroxide solution. Agitate with a mechanical stirrer for 5 minutes. Heat at 80° for 5 minutes, mixing while heating. Cool under water for 1-2 minutes. Add 30 microliters of 1:8 sulfuric acid and 40 microliters of 2% *p*-dimethylaminobenzaldehyde solution in redistilled propanol. Heat at 70° for 20 minutes with constant stirring. Cool in water. Vigorously agitate to mix the contents and

centrifuge for 10 to 15 seconds. Read at 557 $m\mu$. For 2.5-10 micrograms of hydroxyproline, reduce the volume of the reagents to 25%.

*Dental pulp of oxen.*²¹⁷ After aseptic removal from the incisors of oxen, the sample is crushed and soaked in acetone for 24 hours. Dry at 104°. Reflux for 24 hours with 400 times the weight of 1:1 hydrochloric acid. Filter the hydrolyzate and evaporate to dryness. Take up the residue in 25 ml. of water.

Dilute a 0.2-ml. aliquot to 1 ml. Add 1 ml. of 0.25% copper sulfate pentahydrate solution and 1 ml. of 10% sodium hydroxide solution. Warm at 40° for 10 minutes and cool. Add an equal volume of 1:9 sulfuric acid, then 2 ml. of 5% *p*-dimethylaminobenzaldehyde solution in propanol. Warm to 40° for 16 minutes and cool. Read at 555 $m\mu$ against a reagent blank. To convert to collagen, multiply hydroxyproline by 7.46.

By p-dimethylaminobenzaldehyde and sodium peroxide oxidation. In one tube, place 1 ml. of sample containing 0.001-0.02 mg. of hydroxyproline. In a second tube, place 1 ml. of water as a blank. In a third, place a solution containing 0.032-0.113 mg. of hydrolyzed collagen. To each, add 1 ml. of 0.16% copper sulfate pentahydrate solution and 2 ml. of 4.8% sodium peroxide solution. Let stand at room temperature for 5 minutes, shaking occasionally. Mix 1 part of 5% *p*-dimethylaminobenzaldehyde solution in propanol with 2 parts of 1:11 sulfuric acid. Add 6 ml. of this reagent to each tube and shake. Heat at 70° for 20 minutes, cool to room temperature, and read at 550 $m\mu$.

By p-dimethylaminobenzaldehyde and chloramine-T oxidation. Dilute a sample containing 0.01-0.1 micromole of hydroxyproline to 5 ml. and adjust the pH to 8. Add 2 ml. of 5.3% sodium pyrophosphate solution at pH 8 and oxidize the solution at room temperature with 1 ml. of 0.5% chloramine-T solution in methyl Cellosolve. Stop the oxidation after 20 minutes by addition of 1 ml. of 3.6% sodium thiosulfate solution. Add an excess of sodium chloride. If interfering substances are present, shake the sample vigorously 100 times with 5 ml. of toluene. Centrifuge at a low speed and discard the toluene. Stopper, and heat at 100° for 25 minutes to form pyrrole. Cool, and add 5 ml. of toluene. Stopper, and shake vigorously 100 times. Centrifuge at low speed briefly. To a 4.5-ml. portion of the toluene, add 2 ml. of 5% *p*-dimethylaminobenzaldehyde in propanol and read at 560 $m\mu$ after 15 minutes.

²¹⁷ V. Graziano and G. Amici, *Boll. Soc. Ital. Biol. Sper.* **38**, 1302-4 (1962).

α -AMINO- δ -GUANIDINOXYBUTYRIC ACID, CANAVANINE

A reagent for canavanine is pentaacyanoammonioferrate²¹⁸ (cf. Vol. IV, p. 168). Ascorbic acid, hydroxymethylglyoxal, and cysteine can fade the color by reducing the ferric component of the reagent. This color can be restored by adding more reagent or by adding an oxidizer such as hydrogen peroxide or hydrogen persulfate. When present in large quantities, reducing compounds inhibit color formation. The inhibiting action of creatinine can be overcome by addition of excess reagent.

Procedure—To prepare the phosphate buffer at pH 7, mix 29.54 ml. of 0.8% sodium hydroxide solution and 50 ml. of 2.7% monobasic potassium phosphate solution, and dilute to 500 ml. with water. To 1 ml. of neutral sample containing 0.1-1 mg. of canavanine, add 0.5 ml. of 1% pentaacyanoammonioferrate solution. Dilute to 10 ml. with phosphate buffer at pH 7, shaking during the dilution. Expose to daylight for 40 minutes and compare to standards or read with a green or blue-green filter.

NEURAMINIC ACID, SIALIC ACID

This carbohydrate amine acid is developed by thiobarbituric acid.²¹⁹ The method is appropriate for serum, cerebrospinal fluid, and globulin. It is also developed by orcinol,²²⁰ often as Bial's reagent,²²¹ consisting of 500 ml. of concentrated hydrochloric acid containing 1 gram of orcinol and 25 drops of 10% ferric chloride solution (Vol. II, 2nd Ed., p. 273). This is also applicable to the methyl ester.

Neuraminic acid reacts with 3,5-diaminobenzoic acid in hot dilute hydrochloric acid to give an intense green fluorescence.²²² Hexoses interfere slightly.

Procedure—*Serum*. Add 0.2 ml. to 0.8 ml. of 9% sodium chloride solution. Mix, add 1 ml. of 10% trichloroacetic acid solution, and mix. Heat at 80° for 1 hour and cool. Centrifuge for 10 minutes. Add 0.5 ml.

²¹⁸ W. R. Fearon and E. A. Bell, *Biochem. J.* **59**, 221-4 (1955).

²¹⁹ A. Saifer and S. Gerstenfeld, *Clin. chim. Acta* **7**, 467-75 (1962).

²²⁰ C. Chatagnon and P. Chatagnon, *Ann. biol. clin. (Paris)* **13**, 49-55 (1955).

²²¹ N. M. Papadopoulos and W. C. Hess, *Arch. Biochem. Biophys.* **88**, 167-71 (1960).

²²² H. H. Hess and E. Rolde, *J. Biol. Chem.* **239**, 3215-20 (1964).

of 4% sodium iodate in 30% phosphoric acid. After 20 minutes, add 0.5 ml. of 10% sodium arsenate solution in 1:350 sulfuric acid containing 7% of sodium sulfate. Shake until the initial yellow-brown color is discharged. Add 1.5 ml. of 0.6% thiobarbituric acid in 7% aqueous sodium sulfate solution. Heat at 100° for 15 minutes and cool. Add 2.5 ml. of methoxyethanol and mix. Centrifuge and read the clear layer at 549 m μ against a reagent blank.

ETHYL ESTER OF METHYLDOPA,
METHYL 3-(3,4-DIHYDROXYPHENYL-ALANINE ETHYL ESTER

The test substance is determined by a complex reagent specified below.²²³ The ester is sorbed on a column of cellulose impregnated with a solution of 0.35% of sodium bisulfite and 0.05% of ethylenediaminetetraacetate in 0.1 *M* citrate buffer at pH 4.5. Thereafter, the ester is eluted with butanol.

Procedure—Dilute the sample solution with 1:3 acetic acid. To a 5-ml. aliquot containing 0.05-0.075 mg. of the ester, add 5 ml. of 0.6% 1-(2-chloro-4-nitrophenylazo)-naphthalene-2-sulfonic acid in 1:3 acetic acid. Incubate at 35° for 1 hour and cool. Read at 410 m μ against a reagent blank.

²²³ A. D. Marcus and J. D. DeMarco, *J. Pharm. Sci.* **52**, 402-3 (1963).

CHAPTER 4

PROTEINS

A GREATER-THAN-NORMAL confusion arises in a chapter on proteins. Often, proteins are the sample for determination of one or more amino acids.

A solution containing *siderophilin* (*transferrin*), such as serum or plasma, is adjusted with a phosphate buffer to a pH at which protein is not precipitated, but which dissociates the siderophilin-bound iron. The latter is then read with 2,2',2'' terpyridine (Vol. II, p. 316) at 552 m μ or with sulfonated 4,7-diphenyl-1,10-phenanthroline in the presence of ascorbic acid at 554 m μ .¹

Dentine protein is one of the lesser determinations in this field. A pulverized sample is demineralized in 20% EDTA and heated overnight at 100° in a sealed tube.² Thereafter, it is centrifuged and diluted to an extinction of about 1 at 215 m μ . A reading at 225 m μ is subtracted. Each unit difference is equivalent to 17.1 micrograms of nitrogen per ml.

For *keratose*, wool is first defatted with the benzenemethanol azeotrope. The wool is then agitated for 48 hours at 25° with 1.6% peroxyacetic acid, using 100 ml. per gram of wool. Thereafter, 200 mg. of the oxidized wool is shaken for 24 hours with 20 ml. of 0.08% sodium hydroxide solution and filtered. The α -keratose is developed with bromophenol blue.³ For *myoglobin*, tissue is homogenized in a buffer of low ionic strength. After centrifuging, it is read directly.⁴

TOTAL PROTEINS

The biuret complex is formed by a cupric ion with the acid imino groups of two peptide linkages in the protein (Vol. IV, p. 170). There are many variations in the copper reagent. A modified reagent consists of cupric hydroxide suspended in potassium hydroxide solution.⁵ With this reagent, the violet color is read at 540 m μ . In microdetermination, the

¹A. L. Schade, *Behringwerk-Mitt.* **1961**, No. 39, 130-50.

²W. G. Armstrong, *Arch. Oral Biol.* **7**, 771-2 (1963).

³L. Dziedoszyński and M. Maciejewska, *Chem. Anal., Warsaw* **9**, 447-52 (1964).

⁴Baltazar Reynafarje, *J. Lab. Clin. Med.* **61**, 138-45 (1963).

⁵P. Salonen and M. Kekki, *Scand. J. Clin. & Lab. Invest.* **12**, 228-34 (1960).

color can be read at a lower wave length of $320\text{ m}\mu$, where the color has a more pronounced but less specific absorption. A reagent high in alkalinity and low in copper has been used for protein determination of serum without precipitation of cupric hydroxide. The reagent is unstable.

A stable reagent of low alkalinity is prepared with sodium citrate and sodium carbonate combined with the cupric sulfate pentahydrate.⁶ The citrate present in the reagent complexes the excess copper and the reagent is stable at room temperature because of its low alkalinity. More alkalinity is added to the sample with the reagent.⁷ Interferences from turbidity are eliminated by ether extraction of the sample. A second extraction is required for lipemic sera samples. In cases of icteric sera, ether extraction causes high results. Up to 29 mg. of bilirubin per 100 ml. of serum does not interfere.

In concentrations of 25 mg. per 100 ml. in serum containing 7% protein, the bilirubin has an absorbance at $545\text{ m}\mu$ of about 4% of that of the protein-biuret complex when essentially monochromatic light is used. When a wide-band filter is used, the bilirubin has an absorbance of 5-10% of that of the complex. This is determined by addition of cyanide to remove the blue color that appears upon addition of biuret reagent. Sodium sulfite or sodium sulfate do not interfere more than 3% when readings are carried out in square cuvetts. Readings in round cuvetts are higher and may be corrected by addition of the salt to the blank. Ammonium ion does not interfere if the ammonia nitrogen in the sample is no greater than 5 times the protein nitrogen present. A ceiling of under 1% of ammonium sulfate in the final solution should be observed.⁸ Above this concentration, there is a competition for the copper.

A correction is necessary for hemoglobin concentration. The serum protein equivalent of the hemoglobin is calculated by multiplying the hemoglobin concentration by 1.9 and then subtracting this from the apparent serum protein concentration. This method is satisfactory for hemoglobin concentration up to 4%. If hemolysis has occurred, a correction is applied when the hemoglobin concentration is known. Beer's law is followed up to 15% protein concentration at $545\text{ m}\mu$. Beer's law is also followed at $330\text{ m}\mu$, at which micro quantities can be measured with an accuracy of $\pm 1.5\text{ mg. \%}$.

⁶ J. Goa, *ibid.* 5, 218-22 (1953); Richard J. Henry, Charles Sobel and Sam Berkman, *Anal. Chem.* 29, 1491-5 (1957).

⁷ Omar Zafar Hussain, N. S. Shah and S. N. Chaudhuri, *Clin. Chim. Acta* 6, 447-8 (1961).

⁸ P. G. Stanley, *Nature* 197, 1108 (1963).

Other modifications of the biuret reagent contain cupric sulfate pentahydrate, sodium hydroxide, and sodium potassium tartrate to maintain the copper in solution, and potassium iodide to prevent autoreduction.⁹ In some cases, the potassium iodide is eliminated.¹⁰

Solid protein samples, including precipitates with trichloroacetic acid or those formed by heat coagulation, are extracted with half-strength alkaline copper tartrate biuret reagent containing 1.6% of sodium hydroxide¹¹ (cf. Vol. IV, p. 180). This modification is applicable to bacteria, algae, leaf tissue, and yeast. Algae and leaf tissue are extracted with ethanol to remove pigments. Yeast and some algae are pretreated with perchloric acid after ethanol extraction. The combined loss of protein due to this pretreatment is less than 10% (cf. Vol. IV, p. 180). There are large numbers of variations on, and adaptations of, the biuret reaction not included in detail here.¹²

Turbidity from concentrated samples is prevented by addition of the chelating agent, disodium ethylenediaminetetraacetate to the biuret

⁹ Paul F. Fleury and Renée Eberhard, *Pharmacien biologiste* **1**, 24-6 (1956); Angelo Burlina and Gianfranco Panizza, *Il lattante* **27**, 104-7 (1956); P. Burtin, D. Doutriaux, J. J. Pocidalo and Mrs. J. Uriel, *Presse med.* **66**, 413-16 (1958).

¹⁰ Allan G. Gornall, Charles J. Bardawill and Maxima M. David, *J. Biol. Chem.* **177**, 751-66 (1949); P. C. Williams, *J. Sci. Food Agr.* **12**, 58-61 (1961).

¹¹ David Racusen and D. B. Johnstone, *Nature*, **191**, 492-3 (1961).

¹² St. Janoušek and Ruth Reiserová, *Casopis Lékařů Českých* **89**, 181-4 (1950); M. I. Plekhan and E. N. Voluiskaya, *J. Gen. Chem. U.S.S.R.* **22**, 2225-35 (1952); E. M. Kozlova, *Sbornik Studenchesk. Rabot Moskov. Tekhnol. Inst. Myasnoi i Molochnoi Prom.* **1954**, No. 2, 19-21; Ryuju Shiomi, *Igaku to Seibutsugaku* (Med. & Biol.) **30**, 147-50 (1954); Susumu Shibata and Wataru Mizuta, *Bull. Yamaguchi Med. School* **2**, 7-9 (1954); Paul F. Fleury and Renée Eberhard, *Ann. biol. clin.* (Paris) **13**, 1-7 (1955); Federica Trezzi and Laura Balin, *Atti accad. nazl. Lincei, Rend., Classe sci. fis., mat. e nat.* **20**, 60-71 (1956); M. Lubran and D. W. Moss, *Clin. Chim. Acta* **2**, 246-51 (1957); J. Krey, K. Banse and E. Hagmeier, *Kiel. Meeressforsch.* **13**, 35-40 (1957); G. Schweer and W. H. Pook, *Arch. Ophthalmol. Graefes* **158**, 387-92 (1957); P. Polimeni, *Riv. inst. scleroterap. ital.* **32**, 456-68 (1957); Hideo Kimoshita, *Rev. Phys. Chem. Japan*, **27**, 71-5 (1957); M. I. Plekhan, *Zhur. Obshchei Khim* **25**, 603-9 (1955); *ibid.* **28**, 3133-42 (1958); Francisco J. Garcia Canturri, *Anales real. acad. farm.* **24**, 115-34 (1958); S. V. Pande, K. K. Tewari and P. S. Krishnan, *Arch. Microbiol.* **39**, 343-50 (1960); M. Kekki and P. Siltanen, *Scand. J. Clin. Lab. Invest.* **12**, 235-8 (1960); R. D. Strickland, L. Freeman and F. T. Gurule, *Anal. Chem.* **33**, 545-52 (1961); A. C. Jennings, *Cereal Chem.* **38**, 467-79 (1961); Alvin J. Puchnev, *ibid.* **38**, 501-6 (1961); P. C. Williams, *J. Sci. Food Agr.* **12**, 58-61 (1961); George L. Ellman, *Arch. Biochem. J.* **3**, 40-8 (1962); A. F. Gaudy, *Ind. Water and Waste*, **7**, 17-22 (1962); M. A. Inchiosa, *J. Lab. Clin. Med.* **63**, 319-24 (1964); E. Primo, A. Casus, S. Barber, and C. Benedito de Barber, *Rev. Agroquim. Technol. Alimentos*, **4**, 102-8 (1964); H. P. Rieder, *Klin. Wochschr.* **42**, 803-6 (1964).

reagent.¹³ The sensitivity of the reaction is decreased. Interference from calcium in cerebrospinal fluid samples is thus eliminated. This reagent may be used for protein concentrations of less than 300 mg. per 100 ml. Ammonium hydroxide is also used in place of sodium hydroxide with cupric sulfate pentahydrate.¹⁴

Protein-complexed copper is determined with sodium diethyldithiocarbamate.¹⁵ The source of the copper may be cupric sulfate, copper phosphate containing gum arabic,¹⁶ or alkaline copper tartrate reagent. The unbound copper is eliminated by reaction with phosphate or with the strong anion exchanger, Dowex 1.¹⁷ Use of Dowex 1 lowers the sensitivity of the reaction but eliminates highly-colored blanks caused by soluble copper phosphate complexes. Turbidity caused by precipitation of insoluble copper diethyl dithiocarbamate is prevented by addition of albumin to the suspension containing Dowex 1. Beer's law is followed up to 0.4 mg. of protein.

Suitable standards for the biuret reaction are commercial peptones such as Difco Protone, Oxo peptones D, E and F, or U.S.P. peptone powder. These are prepared in an 8-10% solution and preserved by steam sterilization or by addition of 0.02% of mercuric cyanate. They are stable for at least 8 months.¹⁸

Total proteins are pretreated with an alkaline copper reagent and determined with a phosphotungstic-phosphomolybdic acid reagent, also known as the Folin-Ciocalteu reagent or Folin's phenol reagent (cf. Vol. IV, p. 173). This reagent is available commercially or may be prepared as described in Volume III, page 116. Use of a smaller volume of more concentrated alkaline copper reagent and a larger volume of more dilute Folin phenol reagent permits postponement of final mixing of reagents, thus enabling a larger number of samples to be analyzed at one time.¹⁹ In this modified rapid method, the samples and reagents are heated at 50° to speed the reaction. The method is applicable to samples

¹³ Harold L. Rosenthal and Helen I. Cundiff, *Clin. Chem.* **2**, 394-400 (1956).

¹⁴ R. Levin and R. Brauer, *J. Lab. Clin. Med.* **38**, 474-80 (1951); Wataru Mizuta, *Igaku to Seibutsugaku* **27**, 185-9 (1953); Susumu Shibata and Wataru Mizuta, *Bull. Yamaguchi Med. School* **2**, 1-6 (1954).

¹⁵ Herman Nielsen, *Acta Chem. Scand.* **12**, 38-43 (1958).

¹⁶ Hermann Mattenheimer, *Z. physiol. Chem.* **316**, 202-8 (1959).

¹⁷ John Westley and James Lambeth, *Biochim. Biophys. Acta* **40**, 364-6 (1960).

¹⁸ F. Rappaport and M. Loew, *Clin. Chim. Acta* **2**, 126-30 (1957).

¹⁹ Gail L. Miller, *Anal. Chem.* **31**, 964 (1959).

containing protein in the range of 0.04-0.2 mg. Automatic recording equipment has been designed for the purpose.²⁰

A semimicromethod for the analysis of total protein in spinal fluid has been developed using the Folin-Ciocalteu reagent and alkaline copper reagent.²¹ The reaction is approximately 100 times more sensitive than the biuret reaction and is applicable to 20-200 mg. of protein per 100 ml. Cyanide interferes.²²

In the determination of protein in cerebrospinal fluid by this reaction, the color is not proportional to the protein concentration, but the deviation from linearity need not affect routine analysis. A pH of 10 is optimal.²³ This method for cerebrospinal fluid is superior to the turbidimetric method using trichloroacetic acid, as it is relatively unaffected by the albumin/globulin ratio of protein.²⁴ If *p*-aminosalicylic acid is present in blood, it reacts as protein. Deduction of a separate determination of it permits reporting the true value for protein.²⁵

Serum dried on cellulose acetate is dyed with 0.2% Ponceau-S in 3% trichloroacetic acid. The washed and dried protein is then dissolved in 0.4% sodium hydroxide solution for reading at 560 m μ .²⁶ Total amino-nitrogen in maize liquor is determinable with naphthoquinone-4-sulfonate²⁷ (cf. Vol. IV, p. 47). Milk protein in meat products is determined by isolating the milk phosphoprotein. Thereafter, the phosphorus is determined.²⁸ By nitration of the tyrosine and tryptophan present, protein is determined in sputum.²⁹ The results agree with the Kjeldahl method.

In the range of 0.03-0.4 mg. per ml., proteins are determined photometrically by reading the wavelength shift of the absorbance maximum of bromophenol blue in the presence of proteins.³⁰ Although the optical

²⁰ Bennie Zak and Jerry Cohen, *Clin. Chim. Acta* **6**, 665-70 (1961).

²¹ Marie A. Andersch, *Am. J. Clin. Pathol.* **33**, 89-91 (1960).

²² K. Valmikinathan and E. R. B. Shanmugasundaram, *Current. Sci.* **31**, 460-2 (1962).

²³ William H. Daughaday, Oliver H. Lowry, Nira J. Rosebrough and William S. Fields, *J. Lab. Clin. Med.* **39**, 663-5 (1952).

²⁴ Rudolph K. Waldman, Leonard A. Krause and Earle K. Borman, *J. Lab. Clin. Med.* **42**, 489-92 (1953).

²⁵ H. P. Reider, *Clin. Chim. Acta* **6**, 671-6 (1961).

²⁶ R. L. Searcy, G. S. Gough and L. M. Bergquist, *Amer. J. Med. Technol.* **29**, 241-4 (1963).

²⁷ B. L. Scallet, *Stärke* **15**, 50-2 (1963).

²⁸ R. Thalacker, *Dtsch. Lebensmitt. Rdsch.* **59**, 111-13 (1963).

²⁹ Kh. P. Leésik and S. I. Beezborodova, *Lab. Delo* 551-3 (1964).

³⁰ P. G. Scheurlen, *Clin. Chim. Acta* **4**, 760-6 (1959); *Klin. Wochschr.* **37**, 304-5 (1959).

density increment of serum albumin is approximately ten times that of γ -globulin, individual serum protein can be estimated with an error of 1%, which is lower than by the biuret method. Proteins and higher peptides can be determined in the presence of smaller protein-degraded bodies, polysaccharides, lipids, and inorganic salts by dyeing the protein on filter paper with bromophenol blue.³¹ As applied to urine, the protein is denatured with mercuric chloride before dyeing. After washing with dilute acid and drying, the dye is eluted and read.³² Bromothymol blue has been analogously used.³³

Wheat protein reacts with the disulfonic acid, Orange-G, at pH 2.2 to form an insoluble complex. The unbound dye is read at 470 m μ as an indirect measure of the protein present. Beer's law is followed.³⁴ Total protein in milk is determined with Orange-G or with Amido Black 10B dye in citric acid buffer solution.³⁵ In the determination of total protein in milk samples by Amido Black 10B, filtration of the sample gives a lower degree of precision than centrifuging.³⁶ This modification of the Amido Black method can be used for rapid estimations, as there is reasonable linearity up to 3.6% of protein. The relationship between the dye and the milk protein is not stoichiometric. An excess of 0.123 mg. of Amido Black per ml. and an excess of 0.25 mg. of Orange-G dye per ml. is required for complete precipitation of the protein. The Amido Black method is more accurate and sensitive than the Orange-G method.³⁷ The reaction is not affected by time or temperature. A pH of less than 4 causes incomplete precipitation of protein. The protein:dye ratio also affects results. When the ratio of total dye to protein is large, results are unreliable. Too small a ratio produces a marked decrease in dye binding capacity. As applied clinically, the Amido Black 10B method gives results

³¹ S. K. Dutta, *Indian J. Med. Research* **46**, 299-305 (1958).

³² B. Tidström, *Scand. J. Clin. Lab. Invest.* **15**, 167-72 (1963).

³³ Carlo Doleini and Biancamaria Doleini, *Atti Accad. Nazl. Lincei Rend., Classe Sci. Fis., Mat. e nat.* **30**, 271-3 (1961); Gwendoline K. Wilkinson and G. N. Wilkinson, *Australian J. Exptl. Biol. Med. Sci.* **38**, 487-98 (1960); L. Działoszynski and W. Bleszynski, *Chem. Anal. Warsaw* **6**, 999-1004 (1961).

³⁴ Doyle C. Udy, *Cereal Chemistry* **33**, 190-7 (1956).

³⁵ K. Steinsholt, *Meierposten* **46**, 259-64, 279-84 (1957); U. S. Ashworth, Rupert Seals and R. E. Erb, *J. Dairy Sci.* **43**, 614-23 (1960); C. Vanderzant and W. R. Tennison, *Food Technol.* **15**, 63-6 (1961); U. S. Ashworth and M. A. Chaudhry, *J. Dairy Sci.* **45**, 952-7 (1962); F. X. E. J. Vanschoebroek, *Neth. Milk Dairy J.* **17**, 12-45 (1963).

³⁶ K. F. Vogt, *S. Afr. J. Agric. Sci.* **5**, 433-37 (1962).

³⁷ K. Shiga, R. Shimizu and H. Hamada, *Nogyo Gijyutsu Kenkyujo Hokoku* **34**, 1-6 (1959).

that are about double those for the sulfosalicylic acid method. This is attributed to the latter not determining glycoproteins.³⁸

Protein at 0.0001-0.02 mg. per microliter of sample is determined by its quenching effect on eosin Y, Acid Red 87.³⁹ For determination of protein in meats, there is good correlation with the Kjeldahl method by the biuret and Orange-G methods, but not by Amido Black.⁴⁰

Milk proteins are separated by electrophoresis on a starch column. They are then read in the ultraviolet at pH 6-7 and pH 1-2, and results in terms of tyrosine and tryptophan are determined by difference.⁴¹

Total protein in serum, spinal fluid, or urine is estimated by precipitation of the protein at pH 1-4 with Naphthalene Black 12B and reading of the unbound dye at 600 m μ .⁴² This reagent is also applicable to globulin.

Protein may be read in the ultraviolet at its maximum absorption at 280 m μ . The presence of ascorbic acid produces an element of unreliability.⁴³ To avoid interferences from nonprotein constituents of samples, nucleic acids, or nucleotides, the total protein is determined from the difference between the absorbances at 215 and 225 m μ .⁴⁴ Beer's law is followed for 0.02-0.1 mg. per ml. Because of their high absorption at 215 m μ , the following buffers cannot be used in 0.1 M concentrations: acetate, succinate, citrate, phthalate, and barbiturate. When used in 0.005 M concentration with proper blanks, these buffers are acceptable.⁴⁵

Protein fractions dissolved in 0.4% sodium hydroxide solution cannot be used because of the high absorption of the solvent. However, when dissolved in 0.02% sodium hydroxide solution, there is no significant interference. Fractionation of proteins with sodium sulfite cannot be used in the ultraviolet method, since the sulfite ion interferes. Saline solution in place of water is used as a diluent to prevent precipitation of globulins.

³⁸ J. Poortmans and E. Van Kerchove, *Clin. Chim. Acta* **8**, 485-8 (1963).

³⁹ Tosisuke Hiraoka and D. Glick, *Anal. Biochem.* **5**, 497-504 (1963).

⁴⁰ J. Torten and J. R. Whitaker, *J. Food Sci.* **29**, 168-74 (1964).

⁴¹ J. B. Murphy and M. W. Kies, *Biochem. et Biophys. Acta* **45**, 382-4 (1960); D. M. Williams, *Photochem. Photobiol.* **1**, 273-5 (1962).

⁴² Claus M. Plum, Leif Hermansen and Ivan Petersen, *Scand. J. Clin. & Lab. Invest.* **7**, Suppl. 18, 34 pp. (1955).

⁴³ H. P. Rieder, *Clin. Chim. Acta* **3**, 455-70 (1958).

⁴⁴ William J. Waddell, *J. Lab. Clin. Med.* **48**, 311-14 (1956); cf. M. P. Tombs, Feherty Souter and N. F. MacLagan, *Biochem. J.* **71**, 13P-14P (1959); M. P. Tombs, K. B. Cooke, F. Souter, and N. F. MacLagan, *Proteides Biol. Fluids, Proc. 7th Colloq., Bruges, Belg.* **1959**, 37-41.

⁴⁵ Joseph B. Murphy and Marian W. Kies, *Biochim. et Biophys. Acta* **45**, 382-4 (1960).

This method is not applicable to urine samples because of interferences. There is a possibility of interferences of drugs and other compounds in cerebrospinal fluid samples. Ammonium sulfate does not interfere.

The proteins may also be read at 210 $m\mu$.⁴⁶ Absorption by nonprotein constituents is corrected by a blank that has been deproteinized by precipitation with ethanol. Although the absorbance is not maximal at 210 $m\mu$, variations in slit width of the spectrophotometer do not effect results. Absorption by the blank increases from 225 to 215 $m\mu$.

The instrument must be checked against deviations from linearity by use of a reference standard of any known protein, such as 6% bovine albumin solution, diluted 1:1500 and 1:3000. Interferences from carboxylic acids, buffer ions, alcohols, bicarbonate, and aromatic compounds are negligible. Error is apparent only when the protein contains exceptionally large amounts of aromatic amino acids, such as gramicidin or insulin. In the absence of interferences, as little as 0.002 mg. of protein can be determined. The results are in agreement with the trichloroacetic acid and sulfosalicylic acid methods, but are not subject to the errors of turbidity formation.

By column chromatography on dextran gel (Sephadex G-50), interfering substances are removed to permit direct reading of the protein.⁴⁷ Serum proteins that have been separated by electrophoresis cannot be estimated in the ultraviolet, since the means and standard deviations of the absorptivities for all fractions at any given wavelength is too great.⁴⁸

Microgram quantities of total protein in normal plasma, serum, and spinal fluid are determined with tetrabromophenolphthalein ethyl ester or its potassium salt.⁴⁹ The color is stable for at least 20 minutes and the optimum pH of the final mixture is 4.7 for spinal fluid samples and 4.1 for serum samples. The optimum concentration of tetraboromophenolphthalein ethyl ester or its potassium salt in spinal fluid samples in methanol is 2.47 mg.%, and in ethanol, 2.30 mg.%. For serum protein, the optimum concentration is 2.30 mg.% for both methanol and ethanol. Beer's law is followed at 25° for spinal fluid samples, but not for serum samples.

⁴⁶ M. P. Tombs, Felicity Souter and N. F. MacLagan, *Biochem. J.* **73**, 167-71 (1959); Newton Ressler and Jesse F. Goodwin, *Am. J. Clin. Pathol.* **38**, 131-4 (1962); D. S. P. Patterson, *Biochim. Biophys. Acta* **86**, 405-7 (1964).

⁴⁷ R. L. Patrick and R. E. Thiers, *Clin. Chem.* **9**, 283-95 (1963).

⁴⁸ R. D. Strickland, P. A. Mack, T. R. Podleski and W. A. Childs, *Anal. Chem.* **32**, 199-202 (1960).

⁴⁹ Morizo Ishidate, Sadasuke Okano and Yutaka Kuwada, *J. Pharm. Soc. Japan* **72**, 982-5 (1952).

Ammonium sulfate produces a turbidity with dilute protein solutions, which is measured at 450 m μ ,⁵⁰ compared to standards,⁵¹ or read in a nephelometer.⁵² To prepare a silver chloride standard for nephelometric determination of protein and protein fractions in serum and blood plasma, mix 1 ml. of 96% ethanol and 1 ml. of concentrated nitric acid. Dilute 1:4 and add 4 ml. of 0.6% sodium chloride solution and 0.5 ml. of 0.034% silver nitrate solution. Maintain at 40° for 10 minutes.⁵³

Prepare a permanent turbidity standard for nephelometric determination of proteins by placing a glass tube 13-15 mm. in inner diameter, open at both ends, in a container of an acetone solution of white cellulose having the consistency of fluid glue. Remove from the solution and allow to dry vertically on a sheet of dry paper. Glue light blue paper to the bottom and calibrate against standard turbidity solutions or protein standards.⁵⁴ Serum proteins are fractionally precipitated in phosphate buffers of varying molarity and compared turbidimetrically with known standards.⁵⁵

A nephelometric method for protein analysis is based on the insolubility of protein in sulfosalicylic acid solutions (Vol. IV, p. 174). The samples must be free of turbidity.⁵⁶ The turbidity produced by the interaction of proteins with sulfosalicylic acid depends on the albumin/globulin ratio.⁵⁷ When trichloroacetic acid is the reagent, the results are independent of this ratio.⁵⁸ The turbidity of the sample may be compared to the turbidity of a standard solution of silver chloride calibrated with a sample of known protein content,⁵⁹ or the turbidity may be read in an appropriate instrument. By this method, 0.001 mg. of desoxyribose nucleic

⁵⁰ I. A. Parfentjev and M. L. Johnson, *Yale J. Biol. Med.* **25**, 94-106 (1952).

⁵¹ A. Fukutani, *et al.*, *Japan Red Cross Med. J.* **8**, 85-96 (1955); cf. Vol. IV, p. 179.

⁵² Nancy S. Rafferty, Arthur G. Tyrol, Jr. and I. A. Parfentjev, *Clin. Chem.* **4**, 185-93 (1958).

⁵³ Yu. A. Kechek, *Izvest. Akad. Nauk Armyan. S.S.R., Biol. i Sel'skokhoz. Nauki* **7**, 73-85 (1954).

⁵⁴ Yu. A. Kechek, *Lab. Delo* **11**, No. 3, 5-7 (1956).

⁵⁵ Yu. A. Kechek and L. V. Semerdzhyan, *Izvest. Akad. Nauk Armyan. S.S.R., Biol. Nauki* **14**, 45-53 (1961).

⁵⁶ W. B. Yeoman, *J. Clin. Pathol.* **8**, 252 (1955); J. G. Alexander and J. Parkes, *J. Clin. Pathol.* **12**, 91 (1959); C. W. Hiatt and Robert Biereau, *Drug Standards* **28**, 137-9 (1960).

⁵⁷ P. Morand and J. Dufau-Casanabe, *Bull. soc. pharm. Marseilles* **9**, No. 13, 53-61 (1955).

⁵⁸ Richard J. Henry, Charles Sobel and Milton Segalove, *Proc. Soc. Exptl. Biol. Med.* **92**, 748-51 (1956); cf. O. Menlemans, *Clin. Chim. Acta* **5**, 757-61 (1960).

⁵⁹ Yu. A. Kechek, *Trudy Erevan. Med. Inst.* **1956**, No. 8, 73-8.

acid gives turbidity equivalent to 0.0001 mg. of protein. Beer's law is followed for 0.002-0.03 mg. of bovine serum albumin per ml. The estimated accuracy of this method is $\pm 20\%$. The reaction of proteins with tannic acid has also been used turbidimetrically.⁶⁰

Total protein has been read fluorimetrically in milk.⁶¹ The greatly diluted sample in citrate-phosphate-urea solution is excited at 280 m μ and read at 340 m μ with very careful temperature control. Dilution 1:10 with 40% urea solution has also been recommended.⁶² Milk proteins are also read in the ultraviolet.⁶³

Amino groups of proteins react with fluorodinitrobenzene. The intense red color with sodium borohydride then serves for their determination (p. 20). Both total protein and albumin in cerebrospinal fluid are read turbidimetrically after precipitation with isopropanol and trichloroacetic acid.⁶⁴ For total protein along with albumin and globulins by precipitation with potassium phosphate, see page 336.

Procedure—Biuret methods. By cupric hydroxide. Samples containing less than 0.7 mg. of protein. To prepare the cupric hydroxide, add 50 ml. of 2% sodium hydroxide solution to 200 ml. of 2.5% cupric sulfate pentahydrate solution. Filter, and wash the precipitate with water until the filtrates are neutral. Wash twice with 95% ethanol and once with diethyl ether. Dry *in vacuo*. Treat the green alkaline cupric hydroxide powder with 10% potassium hydroxide solution, wash twice with ethanol, once with diethyl ether, and dry *in vacuo*. Pulverize the solid blue cupric hydroxide. Mix 150 mg. of cupric hydroxide with 1 liter of 1.1% potassium hydroxide solution. For serum protein, use 0.56% potassium hydroxide solution.

Prepare a standard of 5-7% clear protein solution. Dilute 1:100 and mix a 1-ml. aliquot with 0.5 ml. of 10% trichloroacetic acid solution. Centrifuge for 10 minutes. Decant the supernatant fluid and dry the wall of the tube. Add 3 ml. of reagent.

Mix the sample with half its volume of 10% trichloroacetic acid solution. Centrifuge for 10 minutes. Decant the supernatant liquid and dry the wall of the tube. Add 3 ml. of reagent.

Stopper the standard and sample tubes. Let stand for 2 hours with

⁶⁰ W. Mejlaum-Katzenellenbogen, *Bull. acad. polon. sci., Classe 11*, 3, 171-3 (1955).

⁶¹ K. K. Fox, V. H. Holsinger and M. J. Pallansch, *J. Dairy Sci.* 46, 302-9 (1963).

⁶² P. D'yachenko, *Moloch. Prom.* No. 4, 7-10 (1963).

⁶³ R. Schober, W. Christ and W. Nicklaus, *Milchwissenschaft* 19, 122-4 (1964).

⁶⁴ D. Watson, *Clin. Chem.* 10, 412-16 (1964).

occasional shaking. Centrifuge for 20 minutes. Decant the supernatant layer and read the standard and sample at 330 $m\mu$ or 540 $m\mu$ against 3 ml. of reagent.

Samples containing 0.1-5 mg. of protein. As a reagent, add 750 mg. of cupric hydroxide to 1 liter of 10% potassium hydroxide solution. Grind the sediment gently and use the suspension after 3 hours.

Dilute 0.05 ml. of a standard containing 5-7% of clear protein solution with 1 ml. of water and add 0.5 ml. of 10% trichloroacetic acid solution. Centrifuge for 10 minutes. Decant the supernatant fluid and dry the wall of the tube. Add 5 ml. of reagent.

To a 0.05-ml. sample, add 5 ml. of reagent. If the protein concentration is less than 0.2% or if interferences are present, precipitate the protein prior to addition of the reagent with a final concentration of 3.3% trichloroacetic acid, wash, and resuspend. Stopper standard and sample tubes. Let stand for 20 minutes, stirring occasionally. Centrifuge for 20 minutes and read the standard and sample at 540 $m\mu$ or 330 $m\mu$ against 5 ml. of reagent. For lipemic sera samples, extract with ether prior to centrifuging to eliminate turbidity.

By cupric sulfate in sodium carbonate-sodium citrate. As the biuret reagent, dissolve 17.3 grams of cupric sulfate pentahydrate in 100 ml. of hot water. Dissolve 17.3 grams of sodium citrate dihydrate and 100 grams of anhydrous sodium carbonate in 800 ml. of water by heating. Cool and pour the citrate-carbonate solution into the cupric sulfate solution with stirring. Dilute to 1 liter at room temperature. Any white precipitate that forms in the reagent does not interfere.

Serum. To prepare the protein standard, determine the protein concentration of a clear serum by Kjeldahl analysis, correcting for the non-protein nitrogen, and using the factor 6.25. Dilute the remaining serum 1:25 with physiological saline solution and saturate with benzoic acid as a preservative. Prepare a reagent blank of 5 ml. of 3% sodium hydroxide solution and a standard of 2.5 ml. of protein standard in 2.5 ml. of 6% sodium hydroxide solution.

Rinse a 0.1-ml. sample into 4.9 ml. of 3% sodium hydroxide solution. Alternatively, dilute a 1-ml. sample to 25 ml. with physiological saline solution and mix a 2.5-ml. aliquot with 2.5 ml. of 6% sodium hydroxide solution.

Add 1 ml. of biuret reagent to both the sample and standard. Let stand for at least 15 minutes, and examine for turbidity by looking for

the Tyndall effect. If negative or showing only a trace, read the standard and sample against the reagent blank at 545 $m\mu$. If the sample is turbid, add 3 ml. of ethyl ether to a developed sample. Shake for 30-60 seconds, centrifuge for 5 minutes, and read against an unextracted reagent blank. Apply a correction of -2% .

If the turbidity cannot be cleared after two ether extractions, add 100 mg. of potassium cyanide to the original sample after reading, and read against water after 2 minutes. Subtract this reading from the original reading as a correction. Cyanide added to the biuret mixture after saturation with ether causes interference due to bubbles.

Cerebrospinal fluid. Mix a 1-ml. sample containing 0.1-2 mg. of protein with 4 ml. of 11% trichloroacetic acid solution. After 10 minutes, centrifuge for 15 minutes. Decant the supernatant liquid and allow the tube to drain. Dissolve the precipitate in 4 ml. of 3% sodium hydroxide solution. Add 0.2 ml. of reagent and mix. Prepare a blank by mixing 4 ml. of 3% sodium hydroxide solution with 0.2 ml. of reagent. Read against the blank after 15 minutes at 330 $m\mu$. To convert to mg. %, use the equation $P = 345 D - 7$, in which P is the mg. % of protein and D is the optical density.

Cerebrospinal fluid. Ultramicromethod. Mix a 0.2-ml. sample containing 0.01-0.4 mg. of protein with 0.1 ml. of 26% trichloroacetic acid solution. Centrifuge for 15 minutes and drain the supernatant liquid. Remove droplets of liquid on the walls of the centrifuge tube with a pipet. Dissolve the precipitate in 0.5 ml. of a mixture of 4 ml. of 3% sodium hydroxide and 0.2 ml. of reagent. After 15 minutes, read at 330 $m\mu$ against a blank prepared from sodium hydroxide solution and reagent.

By alkaline copper tartrate. As a reagent, dissolve 1.5 grams of copper sulfate pentahydrate, 6 grams of sodium potassium tartrate, 30 grams of carbonate-free sodium hydroxide, and 1 gram of potassium iodide in water, and dilute to 1 liter. Treat a 2-ml. sample containing 0.2-0.8% of protein with 8 ml. of reagent and read after 30 minutes at 540 $m\mu$.

By cupric sulfate in sodium hydroxide and ammonium hydroxide. Dissolve 1.7 grams of cupric sulfate pentahydrate in a small amount of water. Dilute 150 ml. of concentrated ammonium hydroxide to 300 ml. with water and add to the cupric sulfate solution. Add 100 ml. of saturated sodium hydroxide solution and dilute to 500 ml. with water.

Dilute a 0.1-ml. serum sample with 2 ml. of water. Add 4 ml. of reagent and read after 5-15 minutes at 570 $m\mu$.

*By cupric sulfate and sodium hydroxide.*⁶⁵ To precipitated protein, add 4.5 ml. of 96% ethanol. Heat for 3-5 minutes with constant stirring and centrifuge. Decant the supernatant layer and treat the precipitate with 4.5 ml. of ether. Centrifuge and decant. Place the precipitate in warm water and stir until it becomes friable. Dissolve in 8 ml. of 0.3% sodium hydroxide solution. Add 0.3 ml. of 20% copper sulfate pentahydrate solution. Mix for 20-30 seconds, centrifuge, and read immediately at 540 $m\mu$.

*Casein.*⁶⁶ Stir a 0.5-gram sample every 3-4 minutes over a 20-25-minute interval with 30 ml. of 5% sodium hydroxide solution. Heat at 45-50°, stirring every 2-3 minutes until either a colloidal solution or a uniform mass of fine casein flakes is formed, usually 5-10 minutes. Dilute to 50 ml. with water, mix, and quickly remove a 2-ml. aliquot. To the aliquot, add 8 ml. of water, mix, add 1 ml. of 5% copper sulfate pentahydrate solution. Mix, and add 1 ml. of 30% sodium hydroxide solution. Mix well for 1 minute and read after 90 minutes against a casein standard.

Milk. As the biuret reagent, add 50 ml. of water to 1.5 gram of copper sulfate pentahydrate and 6 grams of sodium potassium tartrate. Slowly add 300 ml. of 10% sodium hydroxide solution containing 1 gram of potassium iodide.

Dilute a 1-ml. sample with 9 ml. of 0.9% salt solution. Mix a 1-ml. aliquot with 1 ml. of 2.5% sodium tungstate solution and 1 ml. of 1:200 sulfuric acid. Centrifuge and decant. Wash the precipitate with 4-5 ml. of absolute ethanol. Centrifuge and decant. Repeat the washing, and wash finally with 2 ml. of diethyl ether. Dilute to 4 ml. with physiological saline solution.

Add 8 ml. of biuret reagent, shake, and let stand for 30 minutes. Read at 540 $m\mu$ against a blank containing 4 ml. of physiological saline solution and 8 ml. of reagent.

*Milk.*⁶⁷ *Total protein.* Mix 1 ml. of whole or diluted milk with 1 ml. of concentrated nitric acid. Boil over a flame for about 10 seconds and

⁶⁵ E. Gachev, *Lab. Delo* **4**, 8-11 (1958).

⁶⁶ A. Marchenko, *Molochnaya Prom.* **18**, 28-9 (1957).

⁶⁷ L. M. Buruiana, *Naturwissenschaften* **45**, 339-40 (1958).

cool. While immersed in water, neutralize with 30% sodium hydroxide and dilute to 10 ml. Read at 430 $m\mu$.

Casein. Precipitate a 1-ml. sample with 2 ml. of 0.1 M acetate buffer for pH 4.7. Centrifuge, and wash the precipitate twice with the buffer. Add 1 ml. of concentrated nitric acid and proceed as for total protein.

As copper diethyldithiocarbamate. To prepare the alkaline copper tartrate reagent, add 10 ml. of 5% copper sulfate pentahydrate solution to 590 ml. of 0.668% sodium hydroxide solution containing 2 grams of sodium potassium tartrate tetrahydrate. Suspend 200-400 mesh Dowex-1, chloride-form, in deionized water at 200 mg. per ml.

Free the sample from salts and free amino acids by dialysis, or precipitate the protein with 0.5 volume of 50% trichloroacetic acid solution. The concentration of salts and amino acids must be less than 0.4 *M*. If the protein is precipitated, centrifuge for 5 minutes and resuspend in 0.5 ml. of water. Dissolve the protein suspension or 0.5 ml. of dialyzed protein in 1 ml. of alkaline copper tartrate reagent. Let stand at least 5 minutes at room temperature. Add 1 ml. of Dowex-1 suspension and 5 ml. of 0.1% sodium diethyldithiocarbamate solution. Mix, centrifuge briefly, and read the supernatant liquid at 446 or 486 $m\mu$, depending on the amount of protein present, against a blank containing 2.5 ml. of water and 5 ml. of sodium diethyldithiocarbamate reagent.

Alternatively,⁶⁸ use an alkaline copper-phosphate reagent. For this, dissolve 2.5428 grams of monopotassium phosphate in 39 ml. of 2.8% potassium hydroxide solution and dilute to 100 ml. Polyhydric phenols and organic acids must be absent from the sample solution. To 10 ml. of sample solution containing around 8 micrograms of aminonitrogen, add 10 ml. of the phosphate reagent. Add 2 ml. of 1.279% solution of cupric chloride dihydrate. Stir for 20 minutes and centrifuge the precipitate. Mix 5 ml. of the supernatant layer with 15 ml. of water and add 0.2 ml. of 2% sodium diethyldithiocarbamate solution. Shake well and read at 413 $m\mu$.

*Wine.*⁶⁹ The protein precipitant contains 5 grams of molybdophosphoric acid, 15 grams of sulfuric acid, 5 grams of sodium sulfate, and

⁶⁸ I. G. Mokhnachev and V. N. Zelinskaya, *Izv. Vyssh. Ucheb. Zavedeniĭ Pishch. Tekhnol.* No. 6, 138-40 (1961).

⁶⁹ W. Diemair and G. Maier, *Z. Lebensmitt. Untersuch.* 118, 148-52 (1962).

0.25 gram of glucose per liter. To a 10-ml. sample, add 10 ml. of the acid precipitation solution and let stand for 15-20 hours. Centrifuge, and decant the clear liquid. Mix the precipitate with 10 ml. of 96% ethanol and recentrifuge. Dissolve the precipitate in 0.5 ml. of 3% sodium hydroxide solution. Add 0.25 ml. of 20% cupric sulfate pentahydrate solution and 9.25 ml. of 3% sodium hydroxide solution. Shake for 1 minute and let stand for 1-2 hours. Centrifuge, filter the supernatant liquid, and read at 530 $m\mu$.

*Pollen of timothy, corn, birch, English plantain, broad-leaved cotton-tail, orchard grass, sour dock, sheep sorrel, hazelnut, and giant ragweed.*⁷⁰ To prepare the glycerosaline reagent, dilute 46% of glycerine and 4% of sodium chloride to 100 ml. with water. Dilute a suspension of sample containing 0.25-1 mg. of protein per ml. 1:10 or 1:20. Extract the fat with petroleum ether.

Dry the suspension and extract with glycerosaline for 72 hours. Pass through a Seitz filter. Slightly acidify the extract with 1:9 hydrochloric acid. Treat with an excess of 10% phosphotungstic acid solution in 1:100 hydrochloric acid. Allow the precipitate to settle and centrifuge. Decant the supernatant layer containing the nonprotein nitrogen. It may be used as a sample for that determination. Dissolve the precipitate in 5% sodium hydroxide solution. Dilute a 1-ml. aliquot to 5 ml. with water. Add 5 ml. of a biuret reagent, mix, and read after 30 minutes at 450 $m\mu$.

Flour or finely ground whole wheat meal. To prepare the cupric sulfate pentahydrate solution, dissolve 20 grams in water and dilute to 500 ml. Dissolve 140.25 grams of potassium hydroxide in water, cool, and dilute to 250 ml. Dissolve 5 grams of sodium potassium tartrate in 1500 ml. of water. To this, add 30 ml. of the potassium hydroxide solution and 60 ml. of the cupric sulfate solution, and mix. Dilute to 2 liters. Sodium hydroxide solution at 40% concentration may be used in place of the potassium hydroxide solution. Do not use after 48 hours.

The sample may be dried at 100° for 12-24 hours. Saturate a 0.5-gram sample with 1 ml. of carbon tetrachloride. Add 50 ml. of the reagent. Stopper, shake vigorously for 10 minutes, and let stand for 1 hour. Shake briefly and centrifuge for 5 minutes. Decant and read the supernatant layer against a reagent blank at 563 $m\mu$ for wheat meal and 550 $m\mu$ for flour.

⁷⁰E. C. Brennan, *Am. J. Hosp. Pharm.* **18**, 190-3 (1961).

By phosphotungstic-phosphomolybdic acid. Cerebrospinal fluid. Dissolve 20 grams of sodium carbonate and 0.5 gram of sodium potassium tartrate in 1 liter of 0.4% sodium hydroxide solution. Mix 45 ml. with 5 ml. of 0.1% cupric sulfate pentahydrate solution as an alkaline copper reagent. Titrate 1 ml. of Folin-Ciocalteu reagent (Vol. III, p. 116) with 0.4% sodium hydroxide solution. On the basis of the titration, dilute the reagent so that 1 ml. is equivalent to 9 ml. of 0.4% sodium hydroxide solution.

Add a 0.2-ml. sample to 10 ml. of the alkaline copper reagent. Mix, and let stand for 15 minutes. Rapidly add 1 ml. of the diluted phenol reagent and mix immediately. Let stand at least 30 minutes and read at 750 $m\mu$. To correct for the color of nonprotein substances, subtract 6 mg. % from the result.

Semimicromethod. To prepare the alkaline copper reagent, mix 50 ml. of 2% sodium carbonate in 0.4% sodium hydroxide solution with 1 ml. of 0.5% cupric sulfate pentahydrate solution in 1% sodium potassium tartrate solution. As the phenol reagent, titrate Folin-Ciocalteu phenol reagent and dilute to 1 N in acidity.

Rinse a 0.2-ml. sample into 2 ml. of water. Add 5 ml. of alkaline copper reagent and let stand for 10 minutes. Add 2.5 ml. of phenol reagent and mix immediately. Read at 600 $m\mu$ against a reagent blank after 30 minutes.

Rapid method. To prepare the alkaline copper reagent, mix 10 parts of 10% sodium carbonate solution in 2% sodium hydroxide solution with 1 part of 0.5% copper sulfate pentahydrate solution in 1% potassium tartrate solution. Add 1 ml. of reagent to a 1-ml. sample in a photometer tube. After 10 minutes, add 3 ml. of a 1:11 mixture of phenol reagent as rapidly as practical. Heat for 10 minutes at 50°, cool to room temperature, and read at 750 $m\mu$.

*Cultures.*⁷¹ To dissolve the cells, prepare an alkaline copper solution by mixing two stock solutions. The first contains 200 grams of sodium carbonate, 40 grams of sodium hydroxide, and 2 grams of sodium potassium tartrate in 10 liters. The second contains 5 grams of cupric sulfate pentahydrate in 1 liter. Mix 50 parts of the first solution with 1 part of the cupric sulfate solution.

Standardize the phenol reagent (Vol. III, p. 116) as follows: Add 1 part of a solution containing 0.05 mg. of crystalline bovine albumin per

⁷¹ Vance I. Oyama and Harry Eagle, *Proc. Soc. Exptl. Biol. Med.* **91**, 305-7 (1956).

ml. to 5 parts of the alkaline copper solution. Distribute 6 ml. of this solution each into 6 cuvetts. Prepare a parallel series using water in place of the protein solution. Dilute the phenol reagent as follows with water: 5:4, 5:5, 5:6, 5:7, 5:8 and 5:9. Rapidly blow 0.5 ml. of one of these dilutions into one cuvet containing the protein and one containing the water. Read after 15, 30, 60, 90, 120, 180 and 240 minutes against the corresponding water control. Repeat with each of the other diluted reagents. The dilution of phenol reagent that reaches the maximum color within one-half hour and remains stable for at least 2 hours is used.

To prepare the isotonic salt solution, dilute a solution of the following to 1 liter with water: 6.8 grams of sodium chloride, 0.4 gram of potassium chloride, 0.2 gram of calcium chloride, 0.1 gram of magnesium sulfate, 0.125 gram of monobasic sodium phosphate, 2.2 grams of sodium bicarbonate, and 1 gram of dextrose.⁷²

Centrifuge an appropriate sample such as 1 ml. Drain the culture flask of medium by inversion and wash the adherent cell layer twice with the isotonic salt solution. After the second wash, invert the flask over clean gauze and allow to drain for 15 minutes. The cells may be stored in the flask. Add 10 ml. of the alkaline copper reagent. After 10 minutes, shake the solution. If the color is not detectable, use 2 ml. for analysis. For a pale-violet solution, use a 0.5 ml. sample. For a deep-violet solution, dilute with 1-2 volumes of the alkaline copper reagent and use a 0.5 ml. portion.

Dilute the appropriate sample to 5 ml. with alkaline copper reagent and add 1 ml. of water. Rapidly add 0.5 ml. of standardized Folin-Ciocalteu reagent with a syringe. Read after 30 minutes at 660 m μ against a reagent blank. Results are expressed in terms of the bovine albumin equivalent of the unknown cell culture. For conversion, see Table 18.

Chlorilla algae.⁷³ As a reagent, mix 1 ml. of freshly prepared 0.5% copper sulfate pentahydrate solution in 1% sodium potassium tartrate solution with 50 ml. of 2% sodium carbonate solution in 0.4% sodium hydroxide solution. Dilute the phenol reagent 1:1 with water. Centrifuge 5 ml. of an algae suspension, containing up to 0.25 mg. of protein per ml. Wash, and add 5 ml. of 4% sodium hydroxide solution. Heat at 100° for 10 minutes. Centrifuge, and dilute the sodium hydroxide extract with 5 ml. of water. To a 1-ml. aliquot, add 5 ml. of alkaline copper reagent

⁷² W. R. Earle, *J. Nat. Cancer Inst.* **4**, 167 (1943).

⁷³ B. R. Hewitt, *Nature* **182**, 246 (1958).

TABLE 18. CONVERSION OF "BOVINE ALBUMIN EQUIVALENT"
OF 8 TISSUE CULTURE STRAINS TO CELL MASS,
NITROGEN, AND NUMBER

Cell line	Multiplying factors to convert bovine albumin equivalent (mg) to		
	Cell mass (mg)	Cell N (mg)	Cell count ($\times 10^6$)
Mouse fibroblast (Earle)	1.4	.18	.12
HeLa (Gey)	1.4	.16	.16
Intestinal epithelium (Henle)	1.5	.18	.16
Human leukemia (Osgood #111)	1.6	.17	.13
#39 "liver" (Chang)	1.6	.19	.18
KB (Eagle)	1.6	.18	.15
Human conjunctiva (Chang)	1.7	.18	.17
"Liver" (Henle)	1.8	.19	.14

and let stand for 10 minutes. Add 0.5 ml. of diluted phenol reagent and read at $750 m\mu$ after 40 minutes.

By sodium naphthoquinone-4-sulfonate. Maize liquor. Take a sample equivalent to about 2.5 grams of solids. Add 1 ml. of 1% aqueous sodium carbonate solution. Dilute with water to 21 ml. Add 4 ml. of 0.5% solution of sodium naphthoquinone-4-sulfonate. Mix, stopper, and set aside in the dark for 2 hours. Add 4 ml. of 5% sodium acetate solution in 50% acetic acid. Add 4 ml. of 4% sodium thiosulfate solution. Mix, dilute to 50 ml., and read at once at $460 m\mu$.

Milk protein in meat products as phosphate. The sample must not have been heated to 100° . Shake a 1-gram sample for 15 minutes with 20 ml. of 95% ethanol. Follow with 5 similar extractions with 3:1 ethanol-chloroform. Centrifuge each extract to recover the residue. Treat the residue with 20 ml. of 10% perchloric acid at 4° for 30 minutes. This removes acid-soluble phosphorus compounds. Treat the residue with 20 ml. of 5% perchloric acid at 90° for 15 minutes to remove nucleic acids. Wash the residue with 5% perchloric acid at room temperature.

To the residue, in a Kjeldahl flask, add 2.2 ml. of 65% perchloric acid and boil for 5.5 hours. If necessary, to complete the digestion, add a few drops of 30% hydrogen peroxide near the end. Cool and dilute to 20 ml. Determine the phosphorus as molybdenum blue, using amilol as the reducing agent (Vol. IIA, p. 560).

By tetrabromophenolphthalein ethyl ester or the potassium salt. To prepare the tetrabromophenolphthalein ethyl ester reagent, dissolve 134 mg. of the indicator in absolute methanol and dilute to 100 ml. The same concentration of tetrabromophenolphthalein ethyl ester potassium salt may be used or bromophthalein magenta-E may be substituted. Dilute 10 ml. of the indicator solution to 100 ml. with absolute methanol.

Spinal fluid. Add a 0.02-ml. sample to 4 ml. of water and maintain at 25°. Add 1 ml. of reagent solution and mix thoroughly. Add 0.5 ml. of 0.025% acetic acid solution and mix. Let stand at 25° for 5 minutes and read at 600 m μ against a reagent blank.

Serum. Dilute a 1-ml. fresh sample to 100 ml. with 0.85% sodium chloride solution. Dilute a 2-ml. aliquot to 100 ml. with water. Follow the procedure for spinal fluid, starting at "Add a 0.02 ml. sample," using a 4-ml. sample and maintaining the mixture at 20° in place of 25°.

By orange-G or amido black 10B. Milk. Use a flow-through cuvet. This is a U-tube arrangement in which the sample flows downward through the absorbance section and upward and out of the instrument. The constricted part of the tube has a light path of approximately 0.75 mm. The Orange-G reagent contains 1 gram of Orange-G, 21 grams of citric acid, and 2.5 ml. of 0.25% propionic acid solution as a mold inhibitor,⁷⁴ diluted to 1 liter. Dilute this solution 1:1 for use. The Amido Black 10B reagent contains 0.75 gram of Amido Black and 21 grams of citric acid diluted to 1 liter.

Mix a measured sample with 25 ml. of dye reagent. After 30 minutes, remove excess dye by filtering for Orange-G samples and centrifuging for Amido Black samples. Decant the clear solution through the flow-through cuvet and read at 475 m μ for Orange-G and 615 m μ for Amido Black samples.

Alternatively,⁷⁴ transfer a 0.05-ml. sample to filter paper and dry. Immerse for 15 minutes in 1% mercuric chloride solution in 95% ethanol. Follow this with 5 minutes in a 0.1% solution of mercuric chloride in 95% ethanol. Dry at room temperature.

As a reagent, dissolve 0.8 gram of Amido Black 10B in 100 ml. of

⁷⁴ K. F. Vogt, *S. Afr. J. Agr. Sci.* **5**, 433-7 (1962); cf. C. W. Raadsvelt, *Intern. Dairy Congress Proc., 15th Congr. London* **3**, 1638-45 (1959); E. H. Langner and V. de Villiers, *S. Afr. J. Agr. Sci.* **6**, 735-6 (1963); E. Haugen, *Bercin. Forsoglab.* No. 239, 71 pp. (1963).

glacial acetic acid, 570 ml. of absolute methanol, and 450 ml. of water. Immerse the paper in this dye solution. Remove excess dye by immersing in the dye solvent without the dye for, successively, 30 seconds, 30 minutes, 45 minutes, and 45 minutes. Dry, and elute the spots by immersing for 20 hours in 100 ml. of a 4% solution of sodium carbonate in 57% methanol. Read at 620 $m\mu$.

A variant is to prepare a reagent containing 0.07% of Amido Black 10B, 0.358% of disodium phosphate dodecahydrate, and 2.1% of citric acid. Mix 1 ml. of milk sample with 20 ml. of the reagent. After 10 minutes, centrifuge for 10 minutes, and read at 560 $m\mu$.⁷⁵

By orange-G. Milled wheat. Dry Orange-G at 80° and recrystallize from 90% ethanol solution. Prepare a buffer at pH 2.2 with 20.7 grams of citric acid monohydrate and 1.44 grams of disodium phosphate dodecahydrate per liter. To prepare the dye solution, dissolve 100 mg of dye in 100 ml. of buffer and add 1 ml. of 10% aqueous thymol per 8 liters of dye solution to prevent mold.

To a 600-mg. sample in a polyethylene centrifuge tube, add 25 ml. of reagent. Stopper, agitate for 15 minutes, and centrifuge for 5 minutes. Read the unbound dye at 470 $m\mu$. Calculate the protein as follows:

$$P = 40.92 - 45.54 C$$

in which P is the per cent of protein, and C is the concentration of unbound dye at equilibrium.

Whole wheat. Follow the procedure for milled wheat samples, using a 500-mg. sample and shaking for 4 hours in place of 15 minutes.

*By sulfosalicylic acid. Barley flour.*⁷⁶ Shake a 1-gram sample with 100 ml. of 0.02% sodium hydroxide solution for 45 minutes and filter. To each of two 1-ml. portions of the filtrate, add 9 ml. of 3% sulfosalicylic acid solution. To a third 1-ml. portion, add 9 ml. of water. Read at 540 $m\mu$.

By bromophenol blue. Mix a 1-ml. sample containing 0.02-0.4 mg. of protein with 4 ml. of 5 mg. % bromophenol-sulfophthalein indicator solution buffered at pH 3 with Sorensen's citrate buffer. Read at 618 $m\mu$ against an indicator blank.

⁷⁵ W. Heidler and G. Heidler, *Arch. Tierarch.* **6**, 176-85 (1963).

⁷⁶ T. M. Enari, M. Nummi and J. Mikola, *Brauwissenschaft* **13**, 91-2 (1960).

On filter paper. To prepare the bromophenol blue reagent, dilute a 0.5% bromophenol blue solution in methanol to a final concentration of 0.05% with water. Put a few drops of protein solution on clean filter paper. Dry, and spray with 10% calcium chloride solution. Dry, and spray with bromophenol blue reagent. Dry, and slowly wash in a water trough at pH 4-4.2 to remove excess dye. Elute the spot with citrate buffer at pH 6.75 containing 20% of methanol for 16 hours. Read at 595 m μ .

Alternatively,⁷⁷ dry the sample on paper and immerse for 20 minutes in 0.2% bromophenol blue in 1:9 acetic acid-ethanol. Wash 3 times with 2% acetic acid. Elute the appropriate spots with 5 ml. of 0.04% sodium hydroxide solution. Read at 590 m μ .

*By amido black 10B. Serum.*⁷⁸ Dilute the sample 1:200. To a 1-ml. aliquot, add 2 ml. of water and 2.5 ml. of a saturated Amido Black solution in 10% acetic acid-90% methanol. Let stand for 10 minutes at 50°. Pour into a funnel containing 4 ml. of 10% acetic acid solution in methanol. Filter and wash with 20 ml. of a solvent containing 10% of acetic acid and 3% of methanol in water. Elute the dye absorbed on the protein precipitate with 10 ml. of 0.4% sodium hydroxide solution and read the eluted dye.

*On filter paper.*⁷⁹ As a color developer, dissolve 0.25 gram of Amido Black B in 50 ml. of methanol, 40 ml. of water, and 10 ml. of glacial acetic acid. A decolorizer solution is the same vehicle with the dye omitted.

Dilute blood serum with two parts of water and apply it to paper. Dry, and place in the staining solution for 15 minutes. Remove, and apply the decolorizer solution. This removes the dye except where protein is present. Again dry. Elute the colored portion of the paper with 0.04% sodium hydroxide solution. The dried serum and the dye are eluted. Read at 578 m μ .

⁷⁷ J. Homolka, J. Masopust and J. Mojžiš, *Časopis lékařů českých* **99**, 78-83 (1960).

⁷⁸ Willi Riechert and Wolfgang Weber, *Arztl. Forsch.* **11**, 515-16 (1955).

⁷⁹ Otto Suel Hinz, *Klin. Wochschr.* **34**, 1034 (1956); H. Goldhammer, *Arztl. Lab.* **5**, 58-61 (1959); cf. M. Heinzel and V. Neuhoft, *Naturwissenschaften* **46**, 146 (1959).

*By orange G. Milk.*⁸⁰ Prepare a buffer for pH 2.2 by adding 20 ml. of 2.8% solution of anhydrous disodium phosphate to 980 ml. of 1.92% citric acid solution. Dissolve 0.05% of Orange-G in this buffer. Mix 0.5 ml. of sample and 25 ml. of dye solution for 5 minutes. Centrifuge for 20 minutes at 3000 rpm. Dilute 2 ml. to 100 ml. with water and read at 470 m μ .

By naphthalene black. Serum or cerebrospinal fluid. To prepare the precipitating reagent, dissolve 37.65 grams of citric acid, 1.136 gram of dibasic sodium phosphate, and 0.6 gram of Naphthalene Black 12B in water and dilute to 1 liter. Dilute a sample 1:100 with 0.9% sodium chloride solution. Add 2 ml. of the precipitating reagent. After 10 minutes, centrifuge for 15 minutes. Dilute 1 ml. of the supernatant fluid to 25 ml. and read at 600 m μ against water.

Urine. To 4 ml. of a centrifuged sample, add 2 ml. of precipitating reagent. Centrifuge after 15 minutes. Dilute 2 ml. of the supernatant liquid to 25 ml. Read against a blank containing 4 ml. of urine and 2 ml. of water from which a 2-ml. aliquot is diluted to 25 ml. with water.

By nitration. Sputum. Homogenize the sample with an equal volume of 0.4% sodium hydroxide solution. Mix 0.5 ml. of the homogenate with 1 ml. of concentrated nitric acid. Boil over a flame for exactly 1 minute and cool. Add 2.5 ml. of 33% sodium hydroxide solution and dilute to 10 ml. with water. Read at 453 m μ .

In the ultraviolet at 215 and 225 m μ . Plasma. Dilute the sample 1:1000 with 0.9% sodium chloride solution to prevent precipitation of globulins. Read at 215 and 225 m μ against the saline solution. If the absorbance at 215 m μ exceeds 1.5, dilute the protein solution further. Subtract the absorbance at 225 m μ from that at 215 m μ . Multiply the difference by 144 to obtain the protein concentration in micrograms per ml.

Cerebrospinal fluid. Dilute the sample 1:10 and follow the procedure for plasma.

In the ultraviolet at 210 m μ . Cerebrospinal fluid. To prepare the blank, dilute a 0.2-ml. sample with 3 ml. of 95% ethanol and heat at 60° for a few minutes. Centrifuge to precipitate protein. Add 0.2-ml.

⁸⁰ M. Gruener, M. Gentilizza and M. Felajdie, *Kem u. Ind. (Zagreb)* 10, No. 7, 183-9 (1961).

samples to each of two tubes, labeled A and B. Add 3 ml. of 0.9% sodium chloride solution to tube A and 3 ml. of 95% ethanol to tube B. Heat tube B at 60° for a few minutes and centrifuge for 15 minutes. Read tube A against 0.9% sodium chloride solution at 210 m μ . Read the supernatant layer from tube B against the blank. Subtract the optical density of tube B from the optical density of tube A for the corrected reading.

Milk. As a reagent, prepare 4% dodecyl sulfate solution in 24% urea solution adjusted to pH 8 with 4% sodium hydroxide solution. Mix 3 ml. of milk and 3 ml. of reagent. Centrifuge for 20 minutes and remove the fat layer. Dilute 0.5 ml. of the clear aqueous layer with water to 25 ml. Filter through an ultrafilter. Read at 278 m μ against 0.25 ml. of reagent diluted to 25 ml. as a blank.

Turbidimetrically by ammonium sulfate. Serum and plasma. Prepare ammonium sulfate reagents of varying concentrations, as described in Table 19, using gum ghatti as the protective colloid. Adjust the pH of the

TABLE 19. VARIOUS AMMONIUM SULFATE SOLUTIONS USED FOR PRECIPITATION OF SERUM PROTEIN FRACTIONS

<i>% Solution of (NH₄)₂SO₄</i>	<i>(NH₄)₂SO₄ in grams</i>	<i>NaCl in grams</i>	<i>Gum ghatti in grams</i>	<i>Distilled H₂O</i>	<i>Protein fraction precipitated</i>
18	183.67	10.0	5.5	to 1 liter	largely gamma globulin
21	216.07	10.0	5.5	to 1 liter	gamma, beta, alpha globulin
27	270.80	10.0	5.5	to 1 liter	beta, alpha, muco-globulin
50	500.00	10.0	7.0	to 1 liter	albumin

reagent to 7 with 40% sodium hydroxide solution, using 0.02% phenol red indicator. As a preservative, add 25 ppm. of merthiolate to all solutions except the 50% solution. Let the reagent stand overnight and filter before use.

Dilute the sample with 0.1 ml. of physiological saline. Add 10 ml. of reagent according to Table 20. Shake well, and read the turbidity at 450 m μ .

Turbidimetrically by sulfosalicylic acid. Urine. Prepare a standard suspension of silver chloride immediately before use. Mix 1 ml. of 96% ethanol with 1 ml. of 1:4 nitric acid, 1 ml. of 1.4% sodium chloride solu-

TABLE 20. OUTLINE OF PROCEDURE: AMOUNT OF SERUM ANALYZED AT EACH SALT CONCENTRATION

<i>Protein analyzed</i>	<i>(NH₄)₂SO₄ concentration (10 ml.)</i>	<i>Amount of serum analyzed</i>	<i>Preferred dilution</i>	<i>Amount of dilution analyzed</i>
Total protein	50%	0.02 ml.	1-6	0.12 ml.
Total globulin	27%	0.05 ml.	1-3	0.15 ml.
Euglobulin	21%	0.05 ml.	1-3	0.15 ml.
Gamma globulin	18%	0.10 ml.	no dilution	

tion, and 0.5 ml. of 0.34% silver nitrate solution. Keep at 40° for 10 minutes before using in turbidity comparison. Calibrate with a urine sample containing blood serum of known protein content. Add 5 ml. of 3% sulfosalicylic acid solution to a 0.1-ml. urine sample. Compare the turbidity with the silver chloride standard.

Tissue-culture vaccine. Adjust the nephelophotometer with a reference standard containing 0.02 mg. of bovine serum albumin prepared aseptically and treated the same as the sample. Use this standard within one hour. To a 9-ml. sample in a 10 × 150-mm. test tube, add 1.5 ml. of 20% sulfosalicylic acid solution. Stopper, and invert to mix. After 10 minutes, read the relative turbidity by 90° light scattering in a nephelophotometer.

Cerebrospinal fluid. To a 1-ml. sample in a 6 × 5/8" tube, add 4 ml. of 3% sulfosalicylic acid solution. Mix, and let stand for 5 minutes. Put the tube in the right-hand cell of the photometer and put water in the other cell. Match as for hemoglobin estimation with No. 2 eyepiece, a yellow-green filter for 625 mμ. Correct for reading error with a standard wedge. The corrected reading is a direct measure of the concentration of protein in mg. per 100-ml. sample. When the concentration of protein exceeds 120 mg. per 100-ml. sample, dilute the sample with water. Saline as a diluent increases the readings.

Turbidimetrically by trichloroacetic acid. Serum. Dilute a sample 1:400 with 0.85% sodium chloride solution. To a 4-ml. aliquot, add 1 ml. of 12.5% trichloroacetic acid solution and mix immediately. Read the turbidity at 420 mμ from 5-10 minutes after addition of reagents and remixing to remove air bubbles.

Total protein and albumin turbidimetrically. Cerebrospinal fluid. In tubes A, B, and C, take, respectively, 3.8 ml. of isopropanol, 3.8 ml. of a 5% solution of trichloroacetic acid in isopropanol, and 3.8 ml. of 0.9% sodium chloride solution. To each, add 0.2 ml. of cerebrospinal fluid and mix. Centrifuge tubes A and B. Read tube C at 220 m μ against tube A as a blank for total protein. Read tube B against tube A as a blank for albumin.

PROTEIN NITROGEN

A micromethod for protein nitrogen involves determination with ninhydrin of ammonium nitrogen.⁸¹ Use of a citrate buffer stabilizes the color against pH effects and reduces the decrease in color intensity by neutral salts. In the determination of free α -amino nitrogen in tungstic acid filtrates of plasma by ninhydrin, excess ninhydrin and excess cyanide interfere.⁸² The methyl Cellosolve used should give a faint or negative peroxide test with 10% potassium iodide. To prevent deterioration of the methyl Cellosolve, add 0.001-0.01% of hydroquinone or store the methyl Cellosolve under nitrogen. Protein nitrogen may also be measured with Nessler's reagent.⁸³

Sample—Tissue. Follow the procedure under nucleic acid, in Volume IIIA, page 422. Wash the protein residue with 2 ml. of 16% trichloroacetic acid solution. Add 0.5 ml. of 1% selenium dioxide solution in 1:1 sulfuric acid. Evaporate the water by heating on a sand bath and digest the mixture over an open flame until clear. Determine the protein nitrogen, which has been converted to ammonium sulfate, as ammonia with a modified Nessler's reagent.

Protein. Digest a 1-ml. sample containing 0.05-0.1 mg. of nitrogen in a Kjeldahl flask by boiling for 3 hours with 1 ml. of 1:1 sulfuric acid and 3-4 drops of 30% hydrogen peroxide solution. For resistant proteins, add 5 drops of a copper sulfate-potassium sulfate mixture containing 5 grams of copper sulfate pentahydrate and 0.5 grams of potassium sulfate in 100 ml. of water. Cool, and dilute to 20 ml. with water. Add 2 drops of 0.1% methyl red indicator solution and 1.5 ml. of 40% sodium hy-

⁸¹ I. Gordon Fels and Roger Veatch, *Anal. Chem.* **31**, 451-2 (1959).

⁸² Harold Kalant, *ibid.* **28**, 265-6 (1956).

⁸³ John Paul, *Analyst* **83**, 37-42 (1958); Alberto Navarro Zavalia, *Semana Med.* (Buenos Aires) **119**, 1360 (1 page) 1961.

dioxide solution. Neutralize to the methyl red endpoint with 4% sodium hydroxide solution and dilute to 50 ml. Carry a stock standard solution containing 0.05-0.15 mg. of nitrogen as ammonium sulfate and a reagent blank throughout the entire procedure. Develop with ninhydrin.

Procedure—*By Nessler's reagent.* Dilute with water so that 1 ml. contains 0.01 mg. of nitrogen. To a 2-ml. portion, add 2 ml. of a Nessler's reagent prepared by adding a solution of 3.5 grams of gum acacia in 750 ml. of water to a solution of 4 grams of potassium iodide and 4 grams of mercuric iodide in 25 ml. of water and diluting to 1 liter. Add 3 ml. of 8% sodium hydroxide solution and read the yellow color after 15 minutes at 490 m μ .

By ninhydrin. Prepare a citrate buffer by dissolving 26 grams of citric acid and 0.4 gram of stannous chloride dihydrate in 245 ml. of 4% sodium hydroxide solution. Adjust the pH to 5 with 40% sodium hydroxide solution and dilute to 250 ml. with water. Dissolve 4 grams of ninhydrin in 100 ml. of methyl Cellosolve. As the reagent, mix equal volumes of citrate buffer and ninhydrin solution.

To a 1-ml. aliquot of the sample, add 2 ml. of reagent and cover with aluminum foil. Heat at 100° for exactly 20 minutes. Cool immediately in water at 15-20°. Add 5 ml. of a 1:1 1-propanol-water mixture and mix by inversion. Read at 570 m μ .

GLOBULINS

A determination of the tryptophan content of precipitated total protein is taken as a measure of the total globulin.⁸⁴

Addition of 5 volumes of 0.2% 6,9-diamino-2-ethoxyacridone lactate (Rivanol) precipitates all proteins from serum except γ -globulin. This is read at 560 m μ without interference by the Rivanol.⁸⁵ When diluted with collidine-sodium chloride buffer at pH 6.65, only α -globulins in serum react with the cationic surfactant, Octab, to give a turbidity.⁸⁶ Nephelometric methods give a more stoichiometric relationship between concentration and readings than do turbidimetric procedures. The neph-

⁸⁴ Abraham Saifer and Shirley Gerstenfeld, *Clin. Chem.* **8**, 236-41 (1962); cf. *Clin. Chim. Acta* **7**, 149-54 (1962).

⁸⁵ Abraham Saifer and Shirley Gerstenfeld, *Clin. Chim. Acta* **7**, 149-54 (1962).

⁸⁶ Ralph F. Jacox, *J. Lab. Clin. Med.* **37**, 721-7 (1951).

elometric method is more sensitive and allows determination of smaller quantities of α -globulin.⁸⁷

The pH of the medium must be such that the protein carries a charge of the opposite sign to that of the surfactant. In the interaction of serum, α -globulins carry a negative charge at pH 6.65 and the Octab carries a positive charge. The reaction depends on the pH of the medium, the charges of the protein and surfactant, the protein/surfactant weight ratio, temperature, and the ionic strength or salt content of the solution. At higher protein concentrations, the protein-surfactant ratio is less critical. In cases of lower protein concentration, as in cerebrospinal fluid, serum is added to the system. Variations of collidine-sodium chloride buffer from 0.025 *M* to 0.06 *M* have no appreciable effect on the readings. Variation in the final surfactant concentration from 0.005% to 0.02% gives a gradual decrease in readings amounting to 16% between the upper and lower concentrations. Variations of sodium chloride concentration shows little change over the range from 0.05 *M* to 0.1 *M*.

Little or no precipitation occurs in the absence of salt in the system. Losses of α -globulin should be avoided in cerebrospinal fluid samples by not concentrating the protein solution. Albumin does not interfere. Globulins in serum are determined by their tryptophan content. A perchloric acid reagent is applicable without hydrolysis.⁸⁸

Mercuric chloride precipitates γ -globulin resulting in a turbidity which is read in terms of a barium chloride standard.⁸⁹ γ -Globulin has also been estimated turbidimetrically with 0.04% cadmium sulfate in a barbituric acid buffer for pH 7.5.⁹⁰

In solutions alkaline to the isoelectric pH of the protein molecule, the γ -globulin combines with zinc ion to form an insoluble precipitate read at 650 *mμ*. The pH of the solution is critical.⁹¹ Globulins are precipitated by salting out at or near the isoelectric pH with ammonium sulfate⁹² (cf. Vol. IV, p. 186). The redissolved globulin is determined by the biuret method.

Cobalt chloride in a sodium chloride solution at pH 7 precipitates

⁸⁷ Abraham Saifer and Michael E. Zymaris, *Clin. Chem.* 2, 195-213 (1956).

⁸⁸ Abraham Saifer, Shirley Gerstenfeld and Francis Veehsler, *Clin. Chem.* 7, 626-36 (1961).

⁸⁹ Jorge Furio Frattini, *Rev. asoc. bioquim. arg.* 22, 211-16 (1957).

⁹⁰ B. P. Ghosh, *Med. Exptl.* 7, 65-7 (1962).

⁹¹ Harold A. Oberman and Mortan Kulesh, *Am. J. Clin. Pathol.* 39, 519-22 (1958).

⁹² E. Scala and P. Janella, *Boll. soc. ital. biol. spec.* 31, 387-9 (1955); A. Fukutani *et al.*, *Japan Red Cross J.* 8, 85-96 (1955); B. P. Komarova, *Lab. Delo* 9 (12) 27-31 (1963).

γ -globulin to form a turbidity read at 700 m μ .⁹³ Globulin in spinal fluid and blood serum may be developed with Naphthalene Black.

For α , β , and γ globulins determined turbidimetrically by phosphate buffers, see page 341.

Procedure—By Octab. Serum. To prepare the collidine buffer, dissolve 2.64 ml. of 2,4,6-trimethylpyridine in 100 ml. of water. To a 25-ml. aliquot, add 42 ml. of 1:110 hydrochloric acid and 0.467 gram of sodium chloride. Dilute to 100 ml. with water and mix. Adjust the pH to 6.65 with 1:110 hydrochloric acid or 2.64% 2,4,6-trimethylpyridine solution.

To a 0.1-ml. sample, add 15 ml. of 0.468% sodium chloride solution and mix by inversion. To a 1-ml. aliquot of the diluted sample, add 8 ml. of collidine buffer. Adjust the photonephelometer for null point readings. Add 1 ml. of 0.1% octadecyl dimethyl benzyl ammonium chloride (Octab) solution and mix by gentle inversion to avoid air bubbles. Transfer immediately to a cuvet and note the galvanometer deflections. As the reaction proceeds, the hairline indicator deflects to the right and is continually brought back to zero by means of the galvanometer balance knob until the maximum reading is reached. Determine a human serum standard containing 1.08 grams per 100 ml. of α -globulins electrophoretically. Dilute, and run nephelometrically in the same way as the sample.

Cerebrospinal fluid. To a 0.5-ml. sample, add 0.5 ml. of a 1:151 dilution of standard serum in 0.468% sodium chloride solution. Add 8 ml. of collidine buffer and mix. Follow the procedure for serum, starting at "Adjust the photonephelometer . . ." Run 0.5-, 0.75- and 1-ml. aliquots of 1:151 diluted serum in the same manner. Calculate:

Nephelometric reading (serum + cerebrospinal fluid)

— (nephelometric reading of 0.5 ml. of serum)

= nephelometric reading of cerebrospinal fluid

Nephelometric reading (cerebrospinal fluid)

Nephelometric reading (standard serum)

$$= \alpha\text{-globulin standard in mg.} \times \frac{100}{0.5}$$

$$= (\alpha_1 + \alpha_2) \text{ globulins in mg. per 100 ml. of cerebrospinal fluid}$$

⁹³ M. Burstein, S. M. Fine and R. Creyssel, *Rev. hematol.* **15**, 311-17 (1960).

In these calculations, the nephelometric reading corresponding to 0.5, 0.75, or 1 ml. standard value is used, depending on which is closest to the unknown value. The serum blank may be eliminated except in cases of lipemic sera. Calculate:

Maximum nephelometric reading of unknown

Maximum nephelometric reading of standard

$\times \alpha$ -globulin standard in grams per 100 ml.

$= (\alpha_1 + \alpha_2)$ globulins in grams per 100 ml. of serum

By mercuric chloride. Serum. To prepare the turbidimetric standard, precipitate 1:15% barium chloride solution with 1:1800 sulfuric acid and read at 650 to 670 $m\mu$ against water. Assign an arbitrary value of 20 turbidity units. To prepare the reagent, dissolve 30 mg. of mercuric chloride and 6.8 grams of sodium acetate in 1 liter of water. To a 0.1-ml. sample without traces of hemolysis, add 6 ml. of reagent. Mix gently and let stand for 30 minutes. Read in terms of the barium chloride standard.

By zinc sulfate. Serum. As a reagent, dissolve 24 grams of zinc sulfate septahydrate, 280 grams of barbitol, and 210 grams of sodium barbitol in water, and dilute to 1 liter. The pH is 7.6. To 6 ml. of reagent, add 0.1 ml. of sample. Let stand for 30 minutes at 25°. Shake and read the turbidity at 650 $m\mu$. Compare with a barium chloride curve constructed with 3 ml. of 1.15% barium chloride solution diluted to 100 ml. with 0.2% ammonium sulfate.

By cobalt chloride. Serum. To a 0.1-ml. sample, add 0.04 ml. of 1% heparin solution. Add 2 ml. of calcium chloride solution to precipitate β -lipoproteins, and mix. To 0.2 ml. of the supernatant liquid, add 0.04 ml. of 0.275% cobalt chloride hexahydrate solution in 2.5% sodium chloride solution at pH 7. Read the turbidity at 700 $m\mu$ after 15 minutes.

By ammonium sulfate. Serum. To prepare the ammonium sulfate reagent, dissolve 50 or 27 grams of ammonium sulfate, 1 gram of sodium chloride, 0.6-0.8 gram of gum arabic, and a trace of thymol in 100 ml. of water. Adjust the pH to 7 and determine the blank turbidity after filtration. To determine total protein, dilute the sample 10 times. Add 5 ml. of 50% reagent to 0.5 ml. of the diluted sample and estimate the turbidity of the precipitate. To determine globulin, follow the procedure for total

protein (p. 329), using 27% ammonium sulfate reagent. The total protein-globulin is equal to the albumin content.

By naphthalene black. Serum or cerebrospinal fluid. Treat a 1-ml. sample with 1 ml. of a saturated ammonium sulfate solution for 2 hours at room temperature. Centrifuge. Wash the precipitate twice with a half-saturated ammonium sulfate solution. Dissolve the precipitate in 0.9% sodium chloride solution and dilute to 1 ml. Follow the procedure for total protein by Naphthalene Black (page 328), starting at, "Add 2 ml. of precipitating reagent . . ."

Total globulin as tryptophan by thioglycolic acid. Cerebrospinal fluid. To prepare potassium persulfate reagent, add 25 ml. of water to 2 grams of potassium persulfate and shake well for 5 minutes. Use the clear supernatant solution. To prepare the stock solution of thioglycolic acid, add 1 ml. of thioglycolic acid to 19 ml. of glacial acetic acid. As a working solution, dilute 1:10 with glacial acetic acid. Prepare the working solution fresh each day.

To each of 1 ml. of sample and 1 ml. of standard, add 1 ml. of 10% trichloroacetic acid solution and mix. Let stand for 10 minutes at room temperature and centrifuge for 10 minutes. Decant the supernatant solution and suspend the precipitate in 0.25 ml. of water. Prepare a reagent blank of 0.25 ml. of water. Add 1.25 ml. of 0.25% glyoxylic acid solution in glacial acetic acid to each and mix by tapping the tubes. Add 1 ml. of 70% perchloric acid and mix by tapping. After 5 minutes at room temperature, add 0.05 ml. of cold-saturated potassium persulfate solution and let stand for 1 minute at room temperature. Add 0.05 ml. of 0.5% thioglycolic acid solution in acetic acid and mix. After 5 minutes at room temperature, centrifuge for 5 minutes. Read at 560 $m\mu$ against the reagent blank set at zero.

As a standard, dilute dried serum standard or pooled serum of known total globulin content 1:100 with 0.9% sodium chloride solution containing 1:10,000 Merthiolate as a preservative.

Serum. Precipitate globulins from a 0.1 ml. sample with 4 ml. of isopropanol. Centrifuge to separate the precipitate and dissolve it in 0.5 ml. of water. Add 2.5 ml. of glacial acetic acid and mix. Add 2 ml. of 70% perchloric acid and mix. After 5 minutes, add 0.1 ml. of cold-saturated potassium persulfate solution. Mix and add 0.1 ml. of 0.5% thioglycolic acid solution. Centrifuge after 5 minutes and read at 440 $m\mu$.

ALBUMIN

The binding capacity with anionic dyes is specific for albumin and permits its determination in the presence of globulin. Albumin is determined with the anionic dye, 2-(4'-hydroxybenzeneazo)-benzoic acid in a buffered solution at pH 6.2.⁹⁴ Beer's law is followed from 1.25 to 6.25 grams of albumin per 100 ml. of sample. The albumin-dye complex can be measured in the presence of excess dye. Other plasma fractions do not interfere. If the dye solution is saturated with chloroform, it inhibits the combination of the dye with the β form of albumin but not with the α form.⁹⁵ The ratio of β to α forms is 2 in normal sera, lower in pathological sera.

Upon addition of albumin to methyl orange, sodium *p*-dimethylamino azobenzene-*p*-sulfonate, the color shifts to yellow.⁹⁶ The results are higher than that obtained by fractionation followed by Kjeldahl analysis. Hemolyzed serum gives low results. Beer's law is followed up to 6 grams of albumin per 100 ml.

A specific, sensitive, and rapid fluorometric method for the determination of human serum albumin is based on the interaction of albumin in alkaline solution with a dye, Vasoflavine, in the presence of an amine buffer system.⁹⁷ Vasoflavine is a sulfonated, methylated, benzothiazole derivative. In alkaline solutions, the dye has an excitation maximum at 360 $m\mu$. In the presence of human serum albumin, the maximum is shifted to 390 $m\mu$. The fluorescent peak for the dye when activated at its excitation maximum of 360 $m\mu$ is at 440 $m\mu$. The peak for the dye-albumin complex when activated at 390 $m\mu$ is 420 $m\mu$.

This method is in good agreement with the salt fractionation procedure with sodium sulfate and sodium sulfite and the methanol fractionation procedure. Diagnostic dyes, bromsulphthalein, and phenolsulfonphthalein do not interfere. Roentgenographic contrast media do not inter-

⁹⁴ David D. Rutstein, Estelle F. Ingenito and William E. Reynolds, *J. Clin. Invest.* **33**, 211-221 (1954); H. P. Chen and H. Sharton, *Amer. J. Clin. Path.* **40**, 651-4 (1963); Jesse F. Goodwin, *Clin. Chem.* **10**, 309-20 (1964).

⁹⁵ Susumu Shibata, Takaoki Miyaji and Kinzaburo Hasagawa, *Igaku to Seibutsu-gaku* **40**, 24-9 (1956); cf. Hiroshi Takahashi and Yasuhiro Ohba, *Bull. Yamaguchi Med. School* **7**, 107-15 (1960).

⁹⁶ J. S. Bracken and I. M. Klotz, *Am. J. Clin. Pathol.* **23**, 1055-8 (1953); C. McDonald and H. W. Gerarde, *Mikrochem. J.* **7**, 57-62 (1963); M. L. Girard, F. Rousselet and J. Canal, *Pharmazien Biologika* **3**, 493-6 (1964); D. Watson and D. D. Nankiville, *Clin. Chim. Acta* **9**, 359-63 (1964).

⁹⁷ Joseph J. Bethel, *Anal. Chem.* **32**, 560-3 (1960).

fere. It is possible that high concentrations of bilirubin will interfere. Albumin-free fractions obtained from alcohol-fractionation of plasma and other serum proteins do not interfere. Dye concentrations over 0.0005 mg. per ml. decrease in sensitivity. The method is applicable to 0.005 ml. samples. Beer's law is followed up to 0.005 mg. of albumin per ml.

When bound by albumin, 1-aniline-naphthalene-8-sulfonic acid is brightly fluorescent. The unbound dye is not fluorescent.⁹⁸ There is an almost linear relationship between the dye binding capacity and the concentration of plasma albumin up to 6 grams of albumin per 100 ml. When added to whole serum, bromophenol blue is bound almost entirely to albumin.⁹⁹

Albumin in serum is determined by biuret reagent.¹⁰⁰ It follows Beer's law for 0.5-5%. Hemolyzed serum is unsuitable. Albumin may be read in the ultraviolet at 215 and 225 m μ after precipitation of globulin as discussed under total protein, page 329. Albumin may also be read in the ultraviolet at 210 m μ . After precipitation of globulin by 27% ammonium sulfate solution, albumin is determined turbidimetrically¹⁰¹ (see Vol. IV, p. 188, and p. 329, under total protein). For albumin turbidimetrically by phosphate buffers, see page 341. For albumin by agar electrophoresis, see page 342. For albumin and globulin by Folin's phenol reagent, see page 344. For albumin turbidimetrically along with total protein, see page 329.

Procedure—By 2-(4'-hydroxybenzeneazo)benzoic acid. *Serum.* To prepare a stock dye solution, dissolve 1 gram of 2-(4'-hydroxybenzeneazo)-benzoic acid in 500 ml. of 0.04% sodium hydroxide solution. To 100 ml. of the stock dye solution, add enough acetate buffer at pH 5 to give a final pH of 6.2. Dilute to 50 ml. with water and check the pH.

To 0.9 ml. of 0.2 M acetate buffer at pH 6.2, add 0.1 ml. of serum and mix by rotation. Add 12.5 ml. of dye solution and dilute to 25 ml. with 0.1% sodium chloride solution. Stopper, invert to mix, and read immediately at 520 m μ against a blank prepared with 0.1 ml. of serum in 0.9 ml. of acetate buffer at pH 6.2 diluted to 25 ml. with 0.1% sodium chloride solution.

⁹⁸ V. H. Rees, J. E. Fildes and D. J. R. Laurence, *J. Clin. Pathol.* **7**, 336-40 (1954).

⁹⁹ S. H. Blondheim, *Bull. Research Council Israel* **6E**, 223-4 (1957).

¹⁰⁰ J. R. Debro, H. Tarver and A. Korner, *J. Lab. Clin. Med.* **50**, 728-32 (1957); Z. F. C. Zachani, *Z. klin. Chem.* **2**, 91-3 (1964); L. I. Slutskii, *Lab. Delo* 526-30 (1964).

¹⁰¹ A. Fukutani et al., *Japan Red Cross J.* **8**, 85-96 (1955).

By Vasoflavine. Serum. To prepare the ethylenediamine-citrate buffer at pH 9, dissolve 21 grams of citric acid monohydrate in 500 ml. of water. Add 10 ml. of 98% ethylenediamine with rapid mixing and dilute to 900 ml. Adjust the pH to 9 with 10% sodium hydroxide solution and dilute to 1 liter.

The stock solution of Vasoflavine contains 0.04 mg. per ml. of water. As a working solution, dilute the stock solution 100-fold with buffer. Compare the fluorescence of the working Vasoflavine solution with a quinine standard solution containing 0.000845 mg. of quinine sulfate per ml. of 1:350 sulfuric acid as follows. Adjust the fluorometer to a reading of 80 with the quinine standard solution, and to a reading of zero with water. With these adjustments, the Vasoflavine working solution should read 78. If not, adjust the concentration to obtain this reading.

To prepare the diluted Vasoflavine solution for microdetermination of albumin, dilute the working solution 2.5-fold with buffer. The primary filter for the incident beam is Corning No. 5874 and the secondary filter consists of a combination of Corning Nos. 3389 and 4308.

Dilute a 0.1-ml. sample to 200 ml. with water. Add 3 ml. of the diluted sample to 10 ml. of Vasoflavine working solution and dilute to 25 ml. with buffer. Mix, and read in a fluorometer. Adjust the fluorometer, using the Vasoflavine working solution to give a reading of 100 and a blank containing no albumin to adjust the lower range of the fluorometer to give a reading of 40 or lower.

Microdetermination. To 90 ml. of diluted Vasoflavine working solution, add 0.005 ml. of serum. Dilute to 100 ml. with the diluted dye, mix, and read in a fluorometer, adjusting the instrument the same as above.

By methyl orange. Serum or plasma. Prepare a citrate buffer by adding 21 grams of citric acid to 1 liter of 0.8% sodium hydroxide solution. Adjust the pH to 3.5 with 1:110 hydrochloric acid. Add 20 ml. of 0.1% methyl orange solution to 1980 ml. of citrate buffer. Add a few crystals of cresol to inhibit fungus growth. To 5 ml. of reagent, add 0.2 ml. of serum or plasma and mix by inversion. Read at 550 $m\mu$ against water.

At 210 $m\mu$. Serum. Add 0.1 ml. of sample to 4.9 ml. of 27.6% ammonium sulfate solution. Centrifuge for 10 minutes. Dilute 0.5 ml. of the supernatant liquid with 9.5 ml. of water and read at 210 $m\mu$.

Serum after precipitation of globulin by ammonium sulfate. See page 335.

By 1-anilinonaphthalene-8-sulfonic acid. Plasma. To prepare the 1-anilinonaphthalene-8-sulfonic acid, reflux 15 grams of 1-naphthylamine-8-sulfonic acid, 23 grams of aniline and 9 grams of aniline hydrochloride for 15 hours at 150°. Extract the product with 1% sodium hydroxide solution and acidify the extract with glacial acetic acid. Remove brown-colored impurities by treatment with activated carbon. Crystallize the purified sulfonic acid by addition of concentrated hydrochloric acid.

As a dye reagent, dissolve 4 mg. of 1-anilinonaphthalene-8-sulfonic acid in an equivalent amount of 0.4% sodium hydroxide solution and dilute to 500 ml. with water. As a standard fluorescent solution dissolve 40 mg. of 1-naphthylamine-6 (7)-sulfonic acid in 100 ml. of 0.1 M borate buffer at pH 9.2 and dilute to 1 liter with water. To prepare the phosphate buffer, mix 6 volumes of 1% monobasic sodium phosphate with 4 volumes of 1% monobasic potassium phosphate.

A photoelectric fluorometer is used to eliminate subjective errors. The equipment consists of a photomultiplier type R.C.A. 931-A operated from a number of 120-volt high-tension dry batteries. A Pye Scalamp galvanometer with a linear scale is used as the measuring instrument. The light for excitation of the fluorescence is provided by a G.E. 80-watt mercury lamp, type MB/V with a clear glass envelope passing through a Chance light filter OX 7 before entering the fluorometer. The fluorescent intensity is measured in a direction at right angles to the direction of excitation by the photomultiplier provided with Chance OY 18 light filter.

Mix 250 ml. of the dye solution with 50 ml. of phosphate buffer and 50 ml. of 0.9% sodium chloride solution. To a 7-ml. portion of this mixture, add a 0.05-ml. sample and mix well. To another 7-ml. portion, add 0.05 ml. of either 4% human plasma albumin solution in 0.9% sodium chloride solution or 4% bovine plasma albumin solution in 0.9% sodium chloride solution. Adjust the voltage across the photomultiplier until the fluorescent intensity of the fluorescent standard corresponds to 67 galvanometer scale units, in which a full scale deflection equals 140 units. Check this adjustment between each pair of fluorometric readings. Measure the fluorescent intensity of the solution containing the standard albumin. The human albumin solution should be 70, and the bovine albumin, 90. Measure the sample solution.

Plasma in the ultraviolet at 215 and 225 m μ . Mix 1 ml. of plasma with 15 ml. of 22.5% sodium sulfate solution by inversion. Add 6 ml. of ether.

shake, and centrifuge to precipitate the globulin. Dilute 1 ml. of the clear albumin solution with 50 ml. of 0.9% sodium chloride solution and read the albumin in the ultraviolet at 215 and 225 $m\mu$ as described under total protein, on page 328. The difference represents globulin.

Cerebrospinal fluid in the ultraviolet at 215 and 225 $m\mu$. Dilute the sample 1:10 and follow the procedure for plasma.

By biuret reagent. Serum. To 0.2 ml. of serum add, dropwise, 4.8 ml. of 0.4% trichloroacetic acid in 97% ethanol. Centrifuge after 5 minutes and decant. Mix 5 ml. of biuret reagent (page 316) with 2.5 ml. of 1.8% solution of sodium chloride in 0.1% sodium hydroxide solution. Add 2.5 ml. of the decantate to this, and mix by inversion at once. After the escape of gas, it must be clear. Read at 546 $m\mu$ against a reagent blank.

GLOBULIN AND ALBUMIN SIMULTANEOUSLY

A turbimetric method for albumin and globulin in serum is based on the differential precipitation of the various fractions with phosphate solutions.¹⁰² The order of addition of reagent and sample must be followed for reproducibility. Without staining, these serum proteins are estimated by direct ultraviolet densitometry of an agar electrophoretic diagram.¹⁰³ Protein fractions are fixed in ethanol-acetic acid in agar gel and dried on cellophane. Barbitol is rinsed out of the gel and does not interfere. Beer's law is followed.

Total albumin and globulin in cerebrospinal fluid is measured as tyrosine with the Folin-Ciocalteu phenol reagent (see p. 322).¹⁰⁴ Albumin, total globulin, and the globulin fractions are determined with the reagent after precipitation by sodium sulfate.¹⁰⁵ Mucoprotein, albumin, and globulin are precipitated from urine by perchloric acid and acetone. The precipitate is developed with alkaline copper tartrate and Folin-Ciocalteu phenol reagent.¹⁰⁶

Procedure—*Total protein, albumin, α -globulin, β -globulin and γ -globulin by precipitation with potassium phosphate.* To prepare the stock

¹⁰² J. C. Aull and Wm. M. McCord, *J. Lab. Clin. Med.* **46**, 476-83 (1955); S. A. Karpyuk, *Lab. Delo* **8**, 33-6 (1962).

¹⁰³ Kazuo Shimao, *J. Biochem.* **49**, 451-5 (1961).

¹⁰⁴ Shirley Sethna and N. V. Tsao, *Clin. Chem.* **3**, 249-56 (1957).

¹⁰⁵ Yoshitake Tsubogo, *Igaku to Seibutsugaku* **23**, 135-7 (1952).

¹⁰⁶ Abraham Seifer and S. Gerstenfeld, *Clin. Chem.* **10**, 321-34 (1964).

phosphate solution at pH 6.5, add 2268 grams of monobasic potassium phosphate to 4000 ml. of a solution containing 335 grams of sodium hydroxide. Mix to dissolve. Cool to room temperature and dilute to 5000 ml. or add water to a final weight of 675 grams. Prepare diluted solutions as follows:

- Solution 1. Dilute 1235 grams of stock phosphate solution to 1 liter.
- Solution 2. Dilute 1000 grams of stock phosphate solution to 1 liter.
- Solution 3. Dilute 785 grams of stock phosphate solution to 1 liter.
- Solution 4. Dilute 625 grams of stock phosphate solution to 1 liter.

Prepare five tubes, the first containing 10 ml. of water and the remaining tubes numbered to correspond to the diluted phosphate solutions. Add 10 ml. of the corresponding phosphate solution to the appropriate tube. Dilute 1 ml. of serum with 1.5 ml. of water and add 7.5 ml. of stock phosphate solution. Mix and add 1 ml. of the mixture to each of the five tubes. Mix gently and read after 15 minutes at 650 m μ . The optical density of tube 1 is proportional to the total protein. Calculate albumin and globulin as follows:

Optical density of tube 1 $\times F$ = total protein

Optical density of tube 1 - optical density of tube 2 $\times F$ = albumin

Optical density of tube 2 - optical density of tube 3 $\times F$ = α -globulin

Optical density of tube 3 - optical density of tube 4 $\times F$ = β -globulin

Optical density of tube 4 $\times F$ = γ -globulin.

To determine F , determine the total protein content of clear normal human serum by any method. Carry the serum through the above procedure, using the water blank and tube 1 in triplicate. Divide the total protein by the average optical density of tube 1 to obtain F .

Albumin and globulin by agar electrophoresis. Determine the optical density of dried agar. For the supporting medium, prepare a 1% agar solution in sodium barbital-hydrochloric acid buffer, pH 8.6 and ionic strength 0.05.¹⁰⁷ The apparatus appears in Figure 8. Immerse 10 \times 28 clean cellophane in barbital sodium-hydrochloric acid buffer at pH 8.6 and ionic strength 0.05 in the agar electrophoresis apparatus. Adhere the cellophane to the bottom glass plate so that no creases or air bubbles remain. Set a plastic frame of internal size 7 \times 22 cm. on the cellophane. Pour 20 ml. of 1% hot agar solution in barbital sodium-hydrochloric

¹⁰⁷ Kazuo Shimao, *J. Biochem.* **47**, 451 (1961).

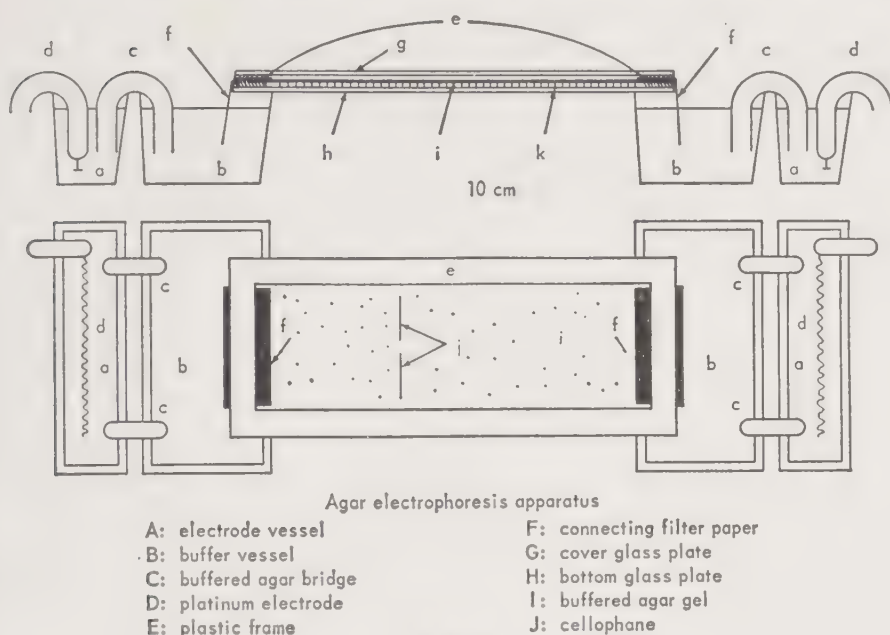


FIG. 8.

acid buffer into the bottom glass plate to make the agar gel 1 mm. thick. After the gel is complete, uniformly apply 0.025-0.03 ml. of serum on a 2×30 -mm. filter-paper strip. Put the strip on the gel 2 cm. to the cathode side from the middle of the gel. Connect the gel and buffer vessels by buffer-saturated filter paper, and place a second plastic frame and cover glass plate as shown in the diagram of the apparatus. Apply 1.5-2 milliamperes of current per cm. width of the gel for 4-5 hours. Serum albumin migrates 5-6 cm. anodically and γ -globulin 2-3 cm. cathodically at room temperature of $15-20^\circ$. When the electrophoretic separation is complete, dip the agar gel with bottom plate and cellophane into 200 ml. of a 9:1 mixture of ethanol and glacial acetic acid as a fixative. The proteins appear as white bands in the gel. After 1 hour, rinse the gel with 200 ml. of 5% acetic acid solution for 15 minutes with occasional shaking. During the rinsing the gel separates from the cellophane.

Prepare a new clean sheet of 10×28 cellophane and immerse in 5% acetic acid solution. Adhere to a clean glass plate the same size as the bottom glass plate, H, leaving no creases or air bubbles. Transfer the gel carefully to the cellophane and set a plastic frame on top to prevent non-uniform shrinkage. Dry the gel in an oven containing a water-filled dish at $40-50^\circ$ for 6 hours. The white protein bands disappear on drying. Cut the dry cellophane to a size of 3×15 cm. and pull it away from the

glass plate. Set in the Type K2 densitometric attachment. Carry out densitometry at 280 $m\mu$ with a band width of 1 $m\mu$.

Cut 1 mm. buffered agar gel into two pieces. Immerse one in protein solution of known concentration and the other in buffer solution for 48 hours. Treat both gel pieces as above and read the optical density of the dried agar gel containing protein against that containing no protein.

Calculate the surface concentration of protein from the volume and thickness of the gel and the volume of the protein solution.

Total albumin and globulin as tyrosine by Folin's reagent. Cerebrospinal fluid. To a 1-ml. sample, add 1 ml. of water and 1 ml. of 10% trichloroacetic acid. Mix by tapping. After 30 minutes, centrifuge until the supernatant layer is clear and the precipitate is well-packed. Aspirate the supernatant fluid.

Dissolve the precipitate in 0.4 ml. of 10% sodium hydroxide solution by rotating. Cover with aluminum foil and heat for 10 minutes at 100°. Cool and transfer quantitatively to a tube. Rinse with water until the total volume is 4.7 ml. Add 0.3 ml. of Folin-Ciocalteu phenol reagent with mixing. Add 5 ml. of water and read after 10 minutes at 520 $m\mu$ against a blank prepared from 0.4 ml. of 10% sodium hydroxide solution, 0.3 ml. phenol reagent, and 9.3 ml. of water. Determine the tyrosine equivalent from a curve prepared from standard tyrosine solution.

Albumin and globulin by biuret. Prepare 4 tubes as follows: Two containing 2 ml. of 28% sodium sulfite solution each, one containing 2 ml. of a mixture of 9 ml. of 28% sodium sulfite solution and 5 ml. of water, and the fourth containing 2 ml. of a mixture of 7 ml. of the sodium sulfite solution and 5 ml. of water. To each tube add 0.1 ml. of serum. To the second, third, and fourth tubes, add 1.5-2 ml. of ether. Shake and centrifuge. Mix 1 ml. of the aqueous portion from each tube with 1 ml. of biuret reagent. Read after 0.5-1 hour at 550 $m\mu$ against 1 ml. of biuret reagent and 1 ml. of water.

Calculate protein fractions as follows:

Albumin	= second tube
Total globulin	= first tube — second tube
α -Globulin	= third tube — second tube
β -Globulin	= fourth tube — third tube
γ -Globulin	= first tube — fourth tube

GLYCOGEN

Glycogen forms a yellowish-brown color when reacted with iodide-iodine reagent. In the presence of calcium chloride, the color of iodine with glycogen is increased tenfold and the absorption is shifted to 460 m μ .¹⁰⁸ Polysaccharides do not interfere. Beer's law is followed up to 1 mg. per ml.

Sample—Tissues. To prepare the iodine-iodide reagent, dissolve 0.26 gram of iodine and 2.6 grams of potassium iodide in 10 ml. of water. To prepare the final reagent, mix 130 ml. of a saturated solution of calcium chloride with 0.5 ml. of the iodine-iodide solution and store in a brown bottle in the refrigerator.

To 0.1 ml. of tissue homogenate or 100 mg. of freshly excised tissue, add 0.9 ml. of 33% potassium hydroxide solution. Heat at 100° for 20 minutes and cool. Remove any cell debris by centrifuging. Add 1.3 ml. of 95% ethanol and mix. Heat to boiling and cool immediately in ice to precipitate the glycogen. Centrifuge at 3000 rpm. for 15 minutes. Pour off the supernatant liquid and drain the tube. To neutralize the excess alkali, add 0.2 ml. of a saturated ammonium chloride solution to the tube and carefully mix with a glass rod. Heat for 5 minutes at 100°. Cool, and add 0.2 ml. of water and 2.6 ml. of iodine reagent. Read at 460 m μ .

CERULOPLASMIN

Ceruloplasmin is the blue copper-containing α_2 -globulin of human plasma and serum. It is determined by its enzymatic oxidation of *p*-phenylenediamine.¹⁰⁹ The optimal pH is 5.4-5.5. Addition of sodium azide to the serum sample causes complete inhibition of the enzymatic activity of the ceruloplasmin; whereas, addition of sodium azide to serum in buffered *p*-phenylenediamine does not completely inhibit this activity. The rate of formation of the colored end product is directly proportional to the concentration over a wide range.

Use of siliconized glassware eliminates error of transfer of small samples and reduces denaturation of diluted samples on glass surfaces. Reproducibility depends on rigid standardization of the procedure. Inter-

¹⁰⁸ Clara R. Krisman, *Anal. Biochem.* **4**, 17-23 (1962).

¹⁰⁹ L. G. Abood, A. Gibbs and E. Gibbs, *A.M.A. Arch. Neurol. and Psychiatry* **77**, 643-5 (1957); Herbert A. Ravin, *J. Lab. Clin. Med.* **58**, 161-8 (1961).

ference is from ascorbate, sodium cyanide, hydroxylamine, semicarbazide, methyl hydrazine, chlorpromazine, and rescinamine, a rauwolfia derivative. Albumin does not interfere and the method is specific for ceruloplasmin. Ceruloplasmin can also be measured by its capacity to oxidize benzidine.¹¹⁰ However, this substrate is less sensitive than phenylenediamine, as it requires a 12- to 24-hour incubation period.¹¹¹

Ceruloplasmin content can be measured by decolorization of potassium cyanide. This is the least accurate method for the metalloprotein and can be used only if the concentration of other copper proteins is low.

Procedure—*By oxidation of p-phenylenediamine dihydrochloride. Serum.* Dissolve p-phenylenediamine dihydrochloride in a minimum of hot water, decolorize with activated carbon and filter while hot. Recrystallize from the water-clear filtrate. Dry the purified white crystals and store *in vacuo* over calcium chloride. Prepare a 0.5% solution.

The sample should be free of hemolysis or turbidity. However, traces of hemolysis or turbidity are compensated for by the use of an enzyme-inhibited control. Use a fresh specimen in which the optical density of a 1:100 dilution in pH 5.4-5.5 buffer exceeds 0.100. Blow a 0.1-ml. sample into the bottom of each of 3 siliconized 18 × 150 mm. test tubes. Add 1 ml. of 0.5% sodium azide solution to one tube as an enzyme-inhibited control. Add 8 ml. of buffer to each tube and 1 ml. of 0.5% p-phenylenediamine dihydrochloride solution as rapidly as possible. Shake to mix and incubate at 37° for 1 hour. Add 1 ml. of 0.5% sodium azide solution to all tubes except the control. Shake to mix and refrigerate for 30 minutes at 4-10° to eliminate rising optical density. Read at 530 mμ. Samples with high readings over the 0.200-0.700 range are diluted with buffer to read.

By decolorization of potassium cyanide. Serum. Bubble carbon dioxide through 3 ml. of serum for 1 minute. Add 0.5 ml. of 5% sodium chloride solution to 2.5 ml. of the carbon dioxide-saturated sample. Read at 610 mμ against water. Adjust 0.1 ml. of 10% potassium cyanide solution to pH 7.8 with hydrochloric acid. Add to the sample. Read every 15 minutes until a maximum decrease is achieved. The difference between the original and final readings, corrected for changes in volume, is a measure of the ceruloplasmin concentration.

¹¹⁰ S. J. Adelstein, T. C. Coombs and B. L. Vallee, *New Eng. J. Med.* **255**, 105-109 (1956).

¹¹¹ Herbert A. Ravin, *J. Lab. Clin. Med.* **58**, 161-8 (1961).

LIPOPROTEINS

Lipids exist in serum in combination with proteins. Almost all plasma lipids can be accounted for in two separate protein fractions that contain largely α - and β -globulins. These lipid fractions are therefore called α - and β -lipoproteins. One method for lipoprotein analysis involves zone electrophoresis on filter paper, using 0.1-0.2 ml. of serum followed by serial cholesterol determinations on the paper segments. The lipoprotein pattern is then constructed from the cholesterol estimation.¹¹² Zone electrophoresis permits isolation of each electrophoretic component in a fairly pure state. An alternative is to apply the Liebermann-Burchard reagent directly to the precipitate.¹¹³ β -Lipoproteins are estimated turbidimetrically by potassium agar,¹¹⁴ or by heparin in the presence of calcium chloride.¹¹⁵ Phosphotungstic acid at a neutral pH and high salt concentration selectively precipitates all low-density lipoproteins in human serum.¹¹⁶

β -Lipoprotein is the only protein of human serum that is precipitated by sulfated amylopectin at pH 8.6. That precipitate is estimated nephelometrically.¹¹⁷ The procedure is applicable up to 1.5 mg. of β -lipoprotein and the method is sensitive to less than 0.1 mg. per sample. The main source of error is interference by extraneous turbid materials such as dust or water-insoluble materials remaining in glassware. Fibrogen also produces a turbidity with sulfated amylopectin, and may interfere. Lower turbidity values are obtained when the reagent is contaminated with sulfated amylose, which occurs when amylose is not completely removed from the amylopectin. Lower results have been reported with amylopectin from wheat as compared with that from potatoes.¹¹⁸

For fluorometric estimation, 1 ml. of serum is mixed with 4 mg. of purified protoporphyrin IX for 1 minute. Excess protoporphyrin is removed by centrifuging. Thereafter, 0.05 ml. of sample is applied to paper strips saturated with barbitone buffer for pH 8.6. Electrophoresis for 16

¹¹² G. S. Boyd, *Biochem. J.* **58**, 680-5 (1954).

¹¹³ R. Fried and J. Hoeflmayr, *Klin. Wochenschr.* **41**, 246 (1963).

¹¹⁴ J. Kellen and E. Pisarcikova, *ibid.* **39**, 1028 (1961).

¹¹⁵ G. Ricci, *Epatologia* **6**, 237-8 (1960); M. Stastny, C. Michalec and E. Novakova, *Acta Univ. Carolinae, Med. Suppl.* **14**, 271-8 (1961); W. G. Dangerfield and G. Faulknee, *Clin. Chim. Acta* **10**, 123-33 (1964).

¹¹⁶ M. Burstein and M. Berlinski, *Rev. franc. etudes clin. et biol.* **6**, 479-81 (1961).

¹¹⁷ Peter Bernfeld, Mordecai E. Berkowitz and Virginia M. Donahue, *J. Clin. Invest.* **36**, 1363-9 (1957).

¹¹⁸ J. Kellen and K. Belaj, *Klin. Wochenschr.* **41**, 200 (1963).

hours at 3 milliamperes follows. Treatment of the segments of the paper carrying the lipoproteins with 1:220 hydrochloric acid at 100° for 20 minutes follows. They are cooled in the dark and fluorescence is read with blue primary and red secondary filters.¹¹⁹

Procedure—*Serum. Zone electrophoresis and cholesterol estimation.* The supporting medium for the electrophoresis run is Whatman 3MM filter paper, 14 × 5 inches. Clamp the paper taut between two 9 × 6 × 1/8-inch glass plates. Allow the lower glass plate to press on a heavy, brass, 8 × 5 × 1/4-inch cooling plate that has an optically flat surface, using a screw clamping device. Solder a long coil of 1/4-inch copper tubing to the lower surface of the cooling plate to permit circulation of water through the apparatus. Cover the entire apparatus with an inverted glass jar. The buffer is 0.04 M diethyl barbituric acid-sodium diethyl barbiturate at pH 8.6. The polyethylene electrode vessels each contain 500 ml. of buffer. The electrodes are 4 × 1/2-inch platinum strips. Equilibrate the hydrostatic pressure in the vessels before each run by means of a glass bridge placed temporarily between the troughs. Draw a pencil line across the filter paper about 4 inches from one end. Spray buffer on this line to produce a damp band about 1 inch wide. Apply a 0.15 ml. serum sample with a drawn-out capillary pipet to the line within 1/4 inch from each end. Spray the rest of the paper with the buffer, leaving dry a 2-inch area at each end. Clamp the paper tightly into the apparatus with thumb screws. Dip the dry end of the paper in buffer. Allow cool water to run through the apparatus for 30 minutes for temperature and hydrostatic equilibrium.

Apply a potential difference of 300 volts across the paper. The current carried by the paper is about 6 milliamperes. Control the air temperature at 12° over a period of an 8-hour run. Remove the paper and let dry in the air for several hours. Mark segments 1 cm. apart parallel to the line of origin and cut a piece 9 × 1/2 inch lengthwise from the center of the paper to be used for protein detection. Cut the paper into 1-cm. pieces, combining corresponding segments, from either side, that had been removed for the protein detection. Insert two 5 × 1-cm. pieces of paper into a centrifuge tube and add 7 ml. of a 1:1 acetone-ethanol mixture. Heat at 30° and gradually increase the temperature to 66°. Heat at 66° for 10 minutes and allow to cool to 40°. Remove the paper with a stainless-steel forceps and wash each strip with a fine jet of the acetone-ethanol mixture.

¹¹⁹ R. L. Searcy, J. L. Korotzer and L. M. Bergquist, *Clin. Chim. Acta* **8**, 148-51 (1963).

adding the washings to the extract. Heat the tubes at 60° and remove the final traces of solvent with a gentle air stream. Place a thin glass rod in each tube and heat in sand in an oven at 110° for 30 minutes.

Treat each tube with 0.5 ml. of glacial acetic acid followed by 1 ml. of a 20:1 freshly prepared ice-cold acetic anhydride-sulfuric acid mixture. Stir, and incubate at 25° in the dark for 30 minutes. Read the cholesterol at $580\text{ m}\mu$. Plot the amount of cholesterol found per segment against the migration distance from the origin to form the lipoprotein pattern. For comparison, the portion of the paper cut out for protein detection is stained with bromophenol blue.

Draw a line on each lipoprotein pattern parallel to the base line in such a manner that both the cholesterol peaks are isolated from as much background material as possible. This line is shown as A-A₁ in Figure 9.

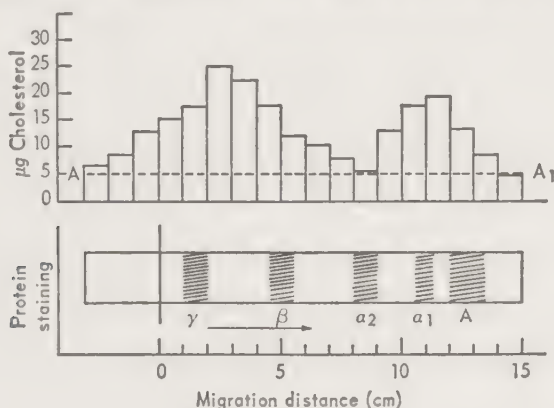


FIG. 9.

Serum lipoprotein pattern contrasted with qualitative protein staining from the same paper

From this datum line, calculate the area under each component. The cholesterol on the α -lipoprotein is expressed as a percentage of the total cholesterol on both the α - and β -lipoproteins. The concentration of each lipoprotein is assessed from the amount of cholesterol associated with it.

By the Liebermann-Burchard reaction. As precipitating reagent, prepare a solution containing 0.28% of anhydrous calcium chloride with 20 units of heparin per ml. To 2 ml. of this add 0.1 ml. of serum. Mix, and after a few minutes, centrifuge. Discard the supernatant layer. Add 2 ml. of 1:5:1 acetic acid-acetic anhydride-concentrated sulfuric acid to the residue. Mix, cool, and store in the dark for 15 minutes. Read at $560\text{--}590\text{ m}\mu$ against a water and reagent blank.

Turbidimetrically by heparin. Serum. Add a 1-ml. sample to 8 ml. of 0.3% calcium chloride solution. Mix, and read at 700 m μ . Add 0.15 ml. of 1% heparin solution and mix. Read after 5 minutes at 700 m μ . The differences of absorbances represents β -lipoprotein.

Alternatively mix 0.2 ml. of serum with 10 ml. of water. Add 0.4 ml. of 0.5% heparin solution and mix. Then mix with 0.2 ml. of 11% solution of calcium chloride hexahydrate and read at 600 m μ .

Turbidimetrically by potassium agar. Serum. To a 0.2-ml. sample, add 3 ml. of 0.066 *M* phosphate buffer at pH 6.8 containing 0.05% of potassium agar. After 5 minutes, read β -lipoproteins at 540 m μ .

By phosphotungstic acid. Serum. As a reagent, dissolve 4 grams of phosphotungstic acid in 14 ml. of normal saline solution. Mix a 0.2-ml. sample with 1.8 ml. of 5% sodium chloride solution and determine the optical density. Add 0.5 ml. of phosphotungstic acid reagent, dilute to 100 ml., and shake. Let stand, and again determine the optical density. The difference is a measure of the removal of lipoprotein.

Nephelometrically by sulfated amylopectin. Serum. Treat corn amylopectin for 5 hours at 70° with chlorosulfonic acid in pyridine to obtain sulfated amylopectin as the potassium salt containing 15-17% of sulfur. Prepare 0.2 *M* tris-hydroxymethylaminomethane buffer and adjust to pH 8.6 with 1:110 hydrochloric acid.

Prepare a reference solution by mixing 10 ml. of 0.3% potassium sulfate solution, 2 ml. of 1:5 hydrochloric acid, and 2 ml. of 10% barium chloride solution. Add 1 gram of polyvinyl alcohol per 100 ml. of solution to maintain the barium sulfate in suspension. Do not use this reference for longer than one hour after preparation. Adjust a photometric nephelometer to 100% transmittance by using a 3/4-inch fused black cell containing freshly prepared turbidity reference solution. Solutions of high polymers can be used as standards after being compared to a freshly prepared barium sulfate standard. Such a standard is a 1% PVM/MA, half amide, type 30, a copolymer of methyl vinyl ether and maleic anhydride (General Aniline & Film Corporation).

Obtain the sample by centrifuging clotted blood. If the sample is markedly turbid, centrifuge for 20 minutes at 20,000 rpm. Dilute a 0.1-ml. sample to 25 ml. with buffer and measure the turbidity in a 3/4-inch fused black cell. To the diluted sample, add 0.15 ml. of 0.1% sulfated amylopectin solution. Mix, and again read the turbidity. The concentration of

β -lipoprotein is expressed as the difference in turbidity between the readings with and without added sulfated amylopectin. This value is called the nephelometric index of β -lipoprotein. The nephelometric index is multiplied by 14.65 to convert it into β -lipoprotein in milligrams per sample. A factor of 12.1 is used to convert it to mg. of β -lipoprotein per 100 ml. of serum, provided that a 0.1-ml. serum sample is used.

MUCOPROTEINS

Mucoproteins are proteins conjugated with hexosamine carbohydrates; they are nonprecipitable by perchloric acid. In serum, the mucoprotein, or seromucoid, amounts to approximately 1% of the total serum protein and is not as easily hydrolyzed as other carbohydrate-containing proteins.

Mucoproteins in serum are isolated by precipitation with phosphotungstic acid after precipitation of other proteins with perchloric acid.¹²⁰ They are then determined by a direct reading of the turbidity, by dissolving the precipitate in biuret reagent,¹²¹ or by application of the Folin-Ciocalteu phenol reagent.¹²² A concentration of 0.4 *M* perchloric acid eliminates contamination by albumin.

After dissolving in biuret reagent, the seromucoids can be separated into four fractions by paper electrophoresis, stained with Amido Black-B, eluted with sodium hydroxide, and read at 620 m μ . The reproducibility is satisfactory and the standard error is $\pm 4\%$.

Mucoproteins are hydrolyzed to release hexosamine, which is developed with *p*-dimethylaminobenzaldehyde in hydrochloric acid.¹²³ Interfering amino acids and sugars are removed by passage through a cation-exchange column. Mucoproteins are isolated from urine by adsorption on benzoic acid and estimated by the purple color formed with acid diphenylamine.¹²⁴ Beer's law is followed up to 3 mg. per 3 ml. of extract. Above this concentration, the curve deviates from a straight line, probably due to the inability of the benzoic acid to adsorb all the mucoprotein present at higher concentrations. After 40 minutes, the purple color turns

¹²⁰ J. Armamendi, *Plasma* 2, 591-5 (1954); J. De la Huerga, Alvin Dubin, Daniel S. Kushner, H. A. Dyniewicz and Hans Popper, *J. Lab. Clin. Med.* 47, 403-8 (1956).

¹²¹ Hiroshi Mizunuma, *Igaku to Seibutsugaku* 38, 3-6 (1956); J. Goa, *Scand. J. Clin and Lab. Invest.* 14, 387-91 (1962).

¹²² J. Gras and J. Capdevila, *Rev. Espan. Fisiol.* 17, 73-9 (1961).

¹²³ E. Nelson McIntosh, *J. Agr. Food Chem.* 9, 421-4 (1961).

¹²⁴ A. J. Anderson and N. F. Maclagan, *Biochem. J.* 59, 638-44 (1955).

blue. Variation in pH is critical. The optimum pH is 4.5. Fructose does not interfere.

Mucoproteins are precipitated from urine by ethanol. After acid hydrolysis the carbohydrate component is determined by resorcinol.¹²⁵ Another technique, after deproteinizing, precipitates with tungstophosphoric acid. The precipitate is then hydrolyzed to hexoses and sialic acid. The hexoses are determined with orcinol (Vol. IIIA, p. 170), reading at 540 m μ . The neuraminic (sialic) acid is developed as the thiobarbituric acid complex (p. 305), extracted with cyclohexanone, and read at 549 m μ . The first is calculated to mucoproteins by multiplying by 8, the latter by 9.43. The orosomucoid content calculated by the two methods is in good agreement.

Procedure—Serum. *Turbidity by phosphotungstic acid.* Dilute a 2-ml. sample with 18 ml. of 0.85% sodium chloride solution. Mix by swirling. While rotating the flask, add 10 ml. of 18% perchloric acid dropwise. Let stand at room temperature for 15 minutes. Filter. The filtrate must be clear. To 15 ml. of the filtrate in a 18 \times 50-mm. test tube calibrated at 6 ml., add 3 ml. of 5% phosphotungstic acid solution in 1:5 hydrochloric acid. Close with a rubber stopper and invert three times. Let stand at room temperature for 20 minutes and centrifuge at 500 rpm. for 5 minutes. Siphon off the supernatant fluid to the 6-ml. mark. Stopper, and invert three times. Read the entire solution at 650 m μ against water.

Barium sulfate cannot be used as a standard, since the ratio of the optical density of barium sulfate to that of mucoprotein varies with the wave length. A suitable standard may be prepared from purified serum mucoprotein treated as for the sample. Another suitable standard is obtained by precipitating bovine serum albumin with phosphotungstic acid. Dilute the bovine serum albumin to contain 0.05-5 mg. of protein nitrogen in 5 ml. of solution. To each diluted standard, add 1 ml. of 5% phosphotungstic acid solution in 1:5 hydrochloric acid. Let stand for 30 minutes, invert three times, and read.

Micromethod. To a 0.5-ml. sample in a 15 \times 125-mm. test tube, add 4.5 ml. of 0.85% sodium chloride solution and 2.5 ml. of 18% perchloric acid solution. Mix by inversion and let stand for 15 minutes. Filter. The filtrate must be clear. Transfer 5 ml. of the filtrate to a cuvet and add 1 ml. of 5% phosphotungstic acid solution in 1:5 hydrochloric acid. Invert, but do not shake. Let stand for 30 minutes and read at 400 m μ .

¹²⁵ A. Ts. Anasashvili, *Lab. Delo*, 9, 26-30 (1963).

against water. Using the micromethod a solution containing 0.2 mg. of bovine albumin nitrogen per 5 ml. gives a turbidity equivalent to serum containing 252 mg. % of mucoprotein.

By the biuret reaction. Serum. To prepare the perchloric acid, dilute 68.4 ml. of 70% perchloric acid to 1 liter with water. Dissolve 173 grams of sodium citrate and 100 grams of anhydrous sodium carbonate in 600 ml. of water. Dilute to 850 ml. Dissolve 17.3 grams of cupric sulfate pentahydrate in 100 ml. of water and dilute to 150 ml. Add the cupric sulfate solution to the citrate-carbonate solution with constant stirring. As the biuret reagent, mix 1 part of this cupric sulfate solution with 20 parts of 3% sodium hydroxide solution.

Dilute a 5-ml. sample with 5 ml. of 0.9% sodium chloride solution. Add 10 ml. of the perchloric acid solution and mix. Filter immediately. To precipitate the total seromucoids, add 1 ml. of 2% phosphotungstic acid solution in 1:5 hydrochloric acid to a 4-ml. portion of the filtrate. Centrifuge. Decant the supernatant liquid and invert the tube on filter paper. Dissolve the precipitate in 4 ml. of biuret reagent and read at 330 $m\mu$ after 15 minutes.

Paper electrophoresis separation of the seromucoids into four fractions. The tris buffer at pH 8.4 for electrophoresis contains 60 grams of hydroxymethylaminomethane and 6 grams of ethylenediaminetetraacetate diluted to 1 liter with water and adjusted to pH 8.4 with hot saturated boric acid solution. For the electrophoresis separation of the seromucoids, add 1 ml. of 2% phosphotungstic acid solution in 1:5 hydrochloric acid to the remaining perchloric acid filtrate of the serum. Centrifuge, and dissolve the precipitate by dropwise addition of 0.4% sodium hydroxide solution to pH 6-7. Add three times its volume of 95% ethanol to reprecipitate the seromucoids, and centrifuge. Decant, and dry the precipitate for 1 minute in a stream of air. Dissolve in a small volume of water. For normal sera, 0.05 ml. is used, and for sera with higher levels of seromucoids, up to 0.2 ml. is used.

Apply 20-40 microliters of this solution on paper electrophoresis strips. Carry out the electrophoresis over 18 hours with a voltage of 3.5 volts per centimeter. Dry the strips at 100° and stain with Anido Black-B. Elute the dye for 2 hours in 0.2% sodium hydroxide solution and read at 620 $m\mu$.

Skeletal muscle. As hexosamine by *p*-dimethylaminobenzaldehyde. The samples are freed from fat and ground and frozen until use. As an

extracting solution, prepare 4.5% potassium chloride solution buffered to pH 8.3 with 0.25% of dibasic sodium carbonate solution. To a 25-gram frozen sample, add 475 ml. of ice-cold extracting solution and glass wool to entrap stromal proteins during extraction. Shake for 3 hours in the cold on a horizontal shaker to remove other hexosamine-containing proteins.

Decant the solubilized proteins with a specially designed separatory funnel. The funnel is equipped with a removable circular piece of 30-mesh stainless steel gauze, 3½ inches in diameter. For each separation, a pad of medium Pyrex No. 800 glass wool approximately 3¾ inches square is fitted snugly under and around the mesh disk. Decant the extract through the mesh-glass wool and discard. Stromal proteins entrapped in the glass wool fragments are retained on the disk. Any small pieces of connective tissue are entrapped by the glass wool pad underneath. Wash thoroughly, add the glass wool pad to the main residue, and carefully remove the residue from the disk. Combine the residue and cover with acetone to extract water and fat. Let stand overnight.

Decant the acetone. Air-dry the residue and hydrolyze in 1:2 hydrochloric acid in stoppered flasks for 4 hours in an autoclave at 15 pounds pressure. Cool, and decant the hydrolyzate. Wash the glass wool mass repeatedly. Add the washings to the hydrolyzate and dilute. Filter.

The ion-exchange column consists of glass tubing 25 cm. × 10 mm. tapered to an opening of 1-2 mm. Place a small amount of glass wool, covered with sand and topped with a circle of filter paper, at the bottom of the column. Wash 250-500-mesh Dowex 50 slowly, several times, with suction with 8% sodium hydroxide solution. Wash with 1:5 hydrochloric acid followed with water. Remove excess moisture with suction. Prepare a 1:1 suspension of the resin in water, shake vigorously, and add 5 ml. of the suspension to the column. Place a filter paper circle on top of the resin.¹²⁶ Run an aliquot of the acid hydrolyzate through the column. Elute the hexosamine with 1:5 hydrochloric acid.

To prepare the developing reagent, recrystallize *p*-dimethylaminobenzaldehyde in hydrochloric acid by addition of a saturated sodium acetate solution. Dissolve 0.8 gram of *p*-dimethylaminobenzaldehyde in 30 ml. of aldehyde-free ethanol. Add 30 ml. of concentrated hydrochloric acid.¹²⁷ To a 4-ml. sample containing 0.02-0.13 mg. of hexosamine, add 1 ml. of freshly prepared 4% acetylacetone in 5.3% sodium carbonate solution. Heat in an oil bath at 92° for 1 hour. Cool, and add 4 ml. of

¹²⁶ Norman F. Boas, *J. Biol. Chem.* **204**, 553-563 (1953).

¹²⁷ P. A. Anastassiadis and R. H. Common, *Can. J. Chem.* **31**, 1093-1107 (1953).

ethanol. Shake thoroughly, and add 1 ml. of *p*-dimethylaminobenzaldehyde reagent. Mix, and read at 540 $m\mu$ after 45 minutes.

By diphenylamine. Urine. As a reagent, dissolve 1 gram of diphenylamine in a mixture of 90 ml. of glacial acetic acid and 10 ml. of concentrated sulfuric acid.

To a 40-ml. sample, add 10 ml. of 10% sulfosalicylic acid solution. Filter after 15 minutes. If the filtrate is not clear, return it to the original flask, bring rapidly to a boil, cool, and refilter. The treatment with sulfosalicylic acid removes heat-coagulable protein and dissolves any phosphates present.

To 40 ml. of the filtrate, add 5 ml. of 20% sodium benzoate solution. Mix, and add 1 ml. of 1:5 hydrochloric acid. Mix, let stand for 15 minutes, and filter on a Hirsch funnel. Drain the precipitate of benzoic acid at the pump. To the precipitate, add 8 ml. of acetone. Stir and centrifuge. Discard the supernatant liquid. Adjust water to pH 9-9.5 with 8% sodium hydroxide solution and add 2 ml. of this water to the residue. Stir for 10 minutes to dissolve the mucoprotein. Add 8 ml. of acetone and 1 drop of a saturated sodium chloride solution. Stir thoroughly, and let stand approximately 10 minutes for complete flocculation. Centrifuge, and discard the supernatant liquid. Treat the residue with two 4-ml. portions of water at pH 9-9.5, centrifuging after each treatment. Combine the supernatant liquids and mix.

The reaction tube is $6 \times \frac{5}{8}$ inch with a vertical groove in the glass stopper to permit equilibrium of pressure with the atmosphere. To a 3-ml. portion of the supernatant fluid in the special tube, add 3 ml. of diphenylamine reagent. Stopper, turning the groove in the stopper to the closed position, and mix. Turn the stopper to the open position and heat at 100° for 30 minutes. Cool under running water and read at 520 $m\mu$. Treat a 40 ml. water blank similarly. For samples containing mucoprotein exceeding 20 mg. per 100 ml., use a 10-20-ml. sample diluted to 40 ml.

By resorcinol. Mix 0.2 ml. of urine with 5 ml. of ethanol. After 20 minutes, centrifuge for 15 minutes. Decant the supernatant layer to waste, and warm the tube at 37° until the ethanol is completely evaporated. Add 1 ml. of water and 5 ml. of 75% sulfuric acid and mix.

As a reagent, dissolve 0.5 gram of resorcinol in 10 ml. of water and add it to 90 ml. of concentrated hydrochloric acid. Add 0.5 ml. of this reagent, mix, and heat at 100° for 10 minutes. Cool, and read at 540 $m\mu$.

Compare with a calibration curve made with equal amounts of galactose and mannose.

FIBRINOGEN

After precipitation with ammonium sulfate, fibrinogen is converted to fibrin with the enzyme thrombin, with or without calcium. The fibrin is then determined. Because of the presence of antithrombin substances in plasma, a thrombin concentration of 5 units per ml. is required to give a maximal fibrin yield from plasma in 1 hour of clotting time. The fibrin can be dissolved in sulfuric acid and developed with Nessler's reagent.¹²⁸ Alcohol is added to the thrombin solution to facilitate collection of fibrin and to aid in suppressing plasma antithrombin activity.

The fibrin clot can be dissolved in 40% urea solution and read at 279 m μ .¹²⁹ Beer's law is followed for 0.3-2 mg. of fibrinogen. After hydrolysis of the fibrin clot in alkali, the protein can be developed by the biuret method or with the phenol reagent.¹³⁰

The turbidity of the fibrin in a barbitone-saline buffer at pH 7.2 is read at 470 m μ .¹³¹ Oxalate cannot be used as the anticoagulant, since calcium oxalate produces its own turbidity. Heparin also interferes. Hemoglobin up to 150 mg. %, urea, lipemia, and bilirubin do not interfere.

The fibrin clot can be dissolved in boiling water and developed with ninhydrin in methyl Cellosolve.¹³² Fibrin may be produced by precipitation of the sample by calcium chloride (cf. Vol. IV, pp. 189-91). The fibrin is dissolved by trypsin in a solution buffered at pH 9, and read in the ultraviolet.¹³³ Fibrin may also be precipitated with calcium chloride, dissolved in biuret reagent with heat, and read at 540 m μ ¹³⁴ or at 750 m μ with a red filter. Fibrinogen is precipitated with ammonium sulfate, and the resulting turbidity is measured at 450 m μ .¹³⁵ Complete precipitation

¹²⁸ H. D. Wycoff, *J. Lab. Clin. Med.* **47**, 645-8 (1956).

¹²⁹ Kjell Jacobsson, *Scand. J. Clin. & Lab. Invest.* **7**, Suppl. 14, 1-54 (1955).

¹³⁰ M. Leclerc and A. Khodabandeh, *Ann. biol. clin.* **11**, 596-8 (1953); S. Gachev and D. Mutavchiev, *Lab. Delo* **4**, 3-7 (1958); Miriam Reiner and Helen L. Cheung, *Clin. Chem.* **5**, 414-20 (1959); T. Strengers and E. G. M. T. Asberg, *Ned. Tijdschr. Geneesk* **107**, 2044-5 (1963).

¹³¹ B. C. Ellis and A. Stransky, *J. Lab. Clin. Med.* **58**, 477-88 (1961).

¹³² Abraham Saifer and Adrienne Newhouse, *J. Biol. Chem.* **208**, 159-79 (1954).

¹³³ L. Korsgaard Christensen, *Scand. J. Clin. & Lab. Invest.* **7**, 246-9 (1955).

¹³⁴ Osvaldo V. Mosto, *Rev. asoc. bioquim. argentina* **19**, 239-41 (1954); R. Ardry, *Ann. Biol. Clin. (Paris)* **18**, 485-8 (1960); G. Ceriotti and L. Spandrio, *Biochim. Appl.* **11**, 111-17 (1964).

¹³⁵ I. A. Parfentjev, M. L. Johnson and E. E. Clifton, *Arch. Biochem. and*

of fibrinogen is accomplished by 12% ammonium sulfate which is 22.8% of saturation. The procedure is applicable to 0.05 to 0.5 gram of fibrinogen per 100 ml. After precipitation with ammonium sulfate, the fibrinogen is dissolved in sodium chloride solution and developed with potassium sodium tartrate biuret reagent.¹³⁶

At a low pH and a high sodium chloride concentration, fibrinogen reacts with the cationic surfactant, Octab, octadecyl dimethyl benzyl ammonium chloride, to produce a measurable turbidity.¹³⁷ Other plasma components are separated from fibrinogen by precipitation with 28.5% ammonium sulfate solution. Precipitated fibrinogen is redissolved in sodium chloride solution. Addition of sodium chloride to the buffer increases the precipitation of fibrinogen with the surfactant to the exclusion of the globulins. It is possible that the surfactant analysis of plasma fibrinogen includes not only clottable fraction but also any unclottable or lysed fibrin. Beer's law is followed for 0.05-3 mg. of fibrinogen.

Procedure—Development with Nessler's reagent. Plasma. To prepare the alcoholic saline solution, mix 100 ml. of 95% ethanol with 900 ml. of 0.85% sodium chloride solution. Dissolve 10 mg. of topical thrombin in 100 ml. of 10% alcoholic saline solution. This solution contains 2.5 units of thrombin per ml. As the ammonium sulfate solution, dissolve 0.472 gram of ammonium sulfate in 1 liter of water.

As the Nessler's reagent, dissolve 22.5 grams of iodine in 20 ml. of water containing 30 grams of potassium iodide. After the solution is complete, add 30 grams of metallic mercury. Shake well. Keep the solution from overheating by periodic immersions in water. Continue to shake until the supernatant layer loses the yellow iodine color. Decant the supernatant layer and test a portion by adding a few drops to 1 ml. of 1% soluble starch solution. Unless the starch test for iodine is obtained, the solution may contain mercurous compounds. To the remaining supernatant layer, add a few drops of iodine solution as above until an excess of free iodine is detected by adding a few drops to 1 ml. of starch solution. Dilute to 100 ml. and mix. To 975 ml. of 10% sodium hydroxide solution, add the entire prepared solution. Mix, and let stand to clear.¹³⁸

Biophys. **46**, 470-80 (1953); A. H. Powell, *Tech. Bull. Registry Med. Technologists* **25**, 48-50 (1955); *Am. J. Clin. Pathol.* **25**, 340-2 (1955); I. I. Dantsig and A. S. Kantorovich, *Lab. Delo* **8**, 22-4 (1962).

¹³⁶ P. Jannella, *Boll. soc. ital. biol. sper.* **33**, 1102-3 (1957).

¹³⁷ Ralph F. Jacox, *J. Lab. Clin. Med.* **44**, 885-9 (1954).

¹³⁸ F. C. Koch and T. L. McMeekin, *J. Am. Chem. Soc.* **46**, 2066-9 (1924).

Centrifuge citrated or oxalated blood for 10 minutes. Pipet 0.1 ml. of plasma into a 15 × 85-mm. tube. Add 2 ml. of thrombin solution and mix quickly. Coagulation of fibrinogen is complete within 3 minutes. Within 1 hour, collect fibrin on a glass needle prepared from a 3-4 mm. glass rod or tubing drawn out to form a 3-inch needle. Collect the fibrin by slowly lowering the tip of the needle into the suspension and rotating it. Wash the fibrin by placing the needle in water for 1-2 minutes, followed by a wash in ethanol for 1-2 minutes. Insert the needle into an 18 × 150-mm. colorimeter tube containing 0.15 ml. of concentrated sulfuric acid. Place the tube in a sand bath on a hot plate and adjust the heat so that the acid refluxes. After 5 minutes, remove from the sand bath and agitate so that all of the fibrin is brought into contact with the acid. Replace in the sand bath and add a drop of 30% hydrogen peroxide solution. Repeat the procedure of heating, agitating, and addition of hydrogen peroxide. Finally, agitate the tube and heat for 5-10 minutes in the sand bath. Remove and cool. Add 6 ml. of water and mix. Add 4 ml. of Nessler's reagent and mix. Read at 500 m μ against a blank containing 1 ml. of water and 4 ml. of Nessler's reagent.

Dissolved in urea and read in the ultraviolet. Plasma. To prepare a buffer at pH 6.1, dissolve 1.9 grams of dibasic sodium phosphate, 6 grams of monobasic sodium phosphate, and 4.3 grams of sodium chloride in 1 liter of water. To prepare the thrombin, dissolve 5000 National Institute of Health units in 10 ml. of 0.9% sodium chloride solution. Separate into 0.2 ml. portions in tubes and store frozen. To prepare the clot solvent, dissolve 400 grams of urea in a solution containing 2.5 grams of dibasic sodium phosphate, 4.4 grams of monobasic sodium phosphate, and 1 gram of sodium chloride per liter, and dilute to 1 liter with that solution.

Place a strip of gauze 7 mm. × 40 mm. against the inner wall of a centrifuge tube and apply 0.25 ml. of 10% disodium ethylenediamine-tetraacetate to the gauze. Dry at 100° for 2 hours. Add 5 ml. of venous blood directly into the tube containing the gauze strip. Immediately invert the tube 8 to 10 times to prevent clotting. Centrifuge for 15 minutes at not less than 4000 rpm in an angle centrifuge. Place 2 ml. of buffer and 0.05 ml. of the thrombin solution in a shallow polyethylene dish 35 mm. in diameter. Pipet 1 ml. of plasma into the dish and let stand for 2 hours at room temperature for complete clotting. Invert the dish into a cloth of fine texture placed on top of a pile of filter papers. Remove the dish and let stand for 5 minutes. Transfer the cloth with the clot to a dish containing 50 ml. of 0.9% sodium chloride solution. After 10 minutes,

loosen one side of the clot from the cloth and wind up the clot on a glass rod. Remove any excess water by carefully pressing the glass rod with the clot against a clean towel. Place the rod and clot in 7.5 ml. of the urea solution. After 40 minutes, stir the dissolved clot with the rod and remove the rod. Read at 279 $m\mu$ against urea solution. If the optical density is higher than 0.8, dilute the fibrin solution.

Development by sodium potassium tartrate biuret reagent. Plasma. The reagent contains 1.5 gram of cupric sulfate pentahydrate, 6 grams of potassium sodium tartrate, 300 ml. of 10% sodium hydroxide solution, and 1 gram of potassium iodide, diluted to 1 liter with water.

To a 0.5-ml. sample, add 0.2 ml. of the thrombin reagent dissolved in 10 ml. of 0.9% sodium chloride solution. Incubate for 5 to 10 minutes at 37°. Add 5-8 ml. of 0.9% sodium chloride solution and centrifuge. Decant the supernatant liquid and wash the precipitate with the sodium chloride solution. Add 1 ml. of 4% sodium hydroxide solution and dissolve the precipitate by heating at 60-70°. Cool to room temperature and add 4 ml. of biuret reagent. Read after 30 minutes at 500 $m\mu$ against a reagent blank.

Development by phenol reagent. Plasma. To prepare the double oxalate solution, dissolve 1.2 grams of ammonium oxalate and 0.8 gram of potassium oxalate in 100 ml. of water. For each 5 ml. of blood, place 0.5 ml. of oxalate solution in a test tube and rotate so that the solution spreads on the walls of the tube as a film. Air dry. As the cupric sulfate solution, dissolve 20 grams of sodium carbonate and 0.5 gram of sodium or potassium tartrate in 1 liter of 0.4% sodium hydroxide solution. Mix 45 ml. of this solution with 5 ml. of 0.1% cupric sulfate pentahydrate solution.

As the phenol reagent, dissolve 100 grams of sodium tungstate and 25 grams of sodium molybdate in 700 ml. of water. Add 50 ml. of 85% phosphoric acid and 100 ml. of concentrated hydrochloric acid. Mix thoroughly. Reflux gently for 10 hours. Remove the condenser and add 150 grams of lithium sulfate. Wash down with 50 ml. of water. After solution is complete, carefully add 4-5 drops of 30% hydrogen peroxide solution and boil for 15 minutes to remove excess peroxide. If the solution is not clear, or is yellow, repeat the treatment with peroxide. Cool to room temperature, dilute to 1 liter, and filter, if necessary, through glass wool. Store in a glass-stoppered brown bottle. Titrate the reagent with 0.4% sodium hydroxide solution to a phenolphthalein endpoint. On the basis of

this titration, dilute the reagent with approximately the sample volume of water to make it 0.9 *N* in acidity.

To prepare the thrombin, dilute a vial of 100 NIH units/ml. to 50 ml. with 0.9% sodium chloride solution. Divide into 0.2-ml. aliquots in 15 × 125-mm. test tubes and keep frozen until use. Thaw the number of thrombin tubes necessary for duplicate tests. Add 5 ml. of 0.9% sodium chloride to each tube and mix well. Add a 5-ml. blood sample to a tube containing 0.5 ml. of dried double oxalate. Mix well to prevent clot formation. Centrifuge for 5 minutes.

Add 0.5 or 1 ml. of plasma to each thrombin tube, and mix quickly and thoroughly. Let stand for 1 hour for complete clotting. Loosen the clot by shaking the tilted tube. If the clot is small, develop with the phenol reagent. If a poor clot is formed, centrifuge, and wash the clot by centrifuging and decanting. Transfer the clot to the middle of a pile of 3 to 4 pieces of hard filter paper, 9 cm. in diameter, which have been placed on top of a few layers of coarse filter paper or paper towels. Cover with a layer of 3 to 4 pieces of hard filter paper and put the coarse paper on top. Remove water by gently placing a water-filled flask on top of the pile. The fluid should be absorbed by the paper in 5 to 10 minutes, leaving a glistening membrane. Loosen the membrane carefully from the paper with a needle or fine-pointed glass rod. Rinse the clot with 0.9% sodium chloride solution and drop into a centrifuge tube calibrated at 5 ml. Add 5 ml. of 1% sodium hydroxide solution and heat at 100° for approximately 15 minutes until the clot dissolves. Cool and dilute to 5 ml. with water.

Add 0.2 ml. of the hydrolyzed fibrin to 10 ml. of the cupric sulfate reagent. Mix, and let stand for 15 minutes. Rapidly add 1 ml. of phenol reagent, and mix immediately and thoroughly. Let stand for 30 minutes and read at 500 *mμ*.

No allowance is made for other proteins in the clot. Albumin or serum may be used instead of fibrinogen as a more convenient protein standard.

Development by ninhydrin. Plasma. To prepare the ninhydrin reagent, dissolve 21 grams of citric acid in 500 ml. of water and add 200 ml. of 4% sodium hydroxide solution. As a preservative, add a few crystals of thymol. Dissolve 67 mg. of anhydrous stannous chloride in 50 ml. of the citrate buffer. Add 2 grams of recrystallized ninhydrin and 50 ml. of methyl Cellosolve, which has been distilled under a vacuum. Stopper, and dissolve by shaking. The final reagent should be yellow and clear. If the reagent is turbid, it may be cleared by placing in warm water.

To prepare the thrombin, dissolve the reagent in 0.9% sodium chloride solution so that 1 ml. is equivalent to 100 NIH units. Dilute 20 ml. of 0.4% sodium hydroxide solution to 100 ml. with 0.9% sodium chloride solution. To prepare the phosphate buffer for pH 6.3, dissolve 0.328 grams of anhydrous dibasic sodium phosphate and 1.385 grams of monobasic potassium phosphate in 500 ml. of water.

Cut untreated, lint-free cloth into 2½-inch-square pieces and boil in water prior to use. Prepare the anticoagulant for the blood sample by dissolving 1.7 grams of glucose, 1.32 grams of sodium citrate, and 0.48 gram of citric acid in water and diluting to 100 ml.

To a 0.25- or 0.5-ml. sample, add 0.5 ml. of phosphate buffer and dilute to 2 ml. with 0.9% sodium chloride solution. Add 0.1 ml. of thrombin reagent and mix. Allow 1 hour for the clot to form. Loosen the clot from the sides of the tube by tapping and let the clot slide onto the lint-free boiled cloth, which is placed on absorbent paper towels. After the clot forms into a thin membrane, wash twice with 0.9% sodium chloride solution and water. To wash, cover the clot completely with the wash fluid and allow it to soak through before adding the next portion of wash. Float the cloth containing the clot in a Petri dish of water and allow the clot to float off by loosening the edges with a pointed glass rod. Roll the clot up on the glass rod and drop into a calibrated photometer tube containing 1.5 ml. of the sodium hydroxide-sodium chloride solution. Dissolve the clot with partial hydrolysis by heating the tube for 30 minutes at 100° with occasional shaking. Cool to room temperature. Add 0.5 ml. of 0.9% sodium chloride solution and 0.5 ml. of ninhydrin reagent. Heat at 100° for 30 minutes with occasional shaking. Cool to room temperature, dilute to 10 ml. with a 1:1 mixture of *n*-propanol and water, and mix well. Centrifuge for 10 minutes at 2000 rpm. to remove any sediment. Clean the outside surface of the tube thoroughly and read at 570 m μ against the propanol solution.

By turbidity. Plasma. To prepare the buffer at pH 7.2, dissolve 2.93 grams of sodium chloride and 5.71 grams of sodium barbitone in 800 ml. of water. Add 22.3 ml. of 1:10 hydrochloric acid and dilute to 1 liter with water. To prepare the calcium-thrombin solution, dilute thrombin to 33.8 NIH units per ml. with 0.85% sodium chloride solution. Mix one volume with an equal volume of 37% calcium chloride solution. Store frozen.

Add a 5-ml. blood sample to 1 drop of 38% sodium citrate solution in a 4 × 0.5-inch tube. Mix, and centrifuge in an angle centrifuge at 3000

rpm. for 10 minutes to separate plasma. Dilute a 1-ml. plasma sample with 11 ml. of buffer, and mix by swirling. Transfer 6 ml. to a photometer tube. Add 2 drops of calcium-thrombin solution to the sample tube. Mix by swirling for 10 to 15 seconds, being careful to avoid air bubbles. Read the turbidity after 20 minutes against the blank in a photometer at 470 $m\mu$.

Alternatively, for the determination, add 0.5 ml. of plasma to 5.5 ml. of buffer and mix by inversion. Remove 3 ml. of the solution for the test and use the remainder for the blank. To the 3 ml. of solution, add 1 drop of calcium-thrombin solution and mix by inversion for 10-15 seconds. After 20 minutes, read the turbidity against the blank at 470 $m\mu$.

Precipitation by calcium chloride and fibrin development with biuret reagent. Plasma. Add a 1-ml. sample to 50 ml. of 0.9% sodium chloride solution. Add 0.8 ml. of 5% calcium chloride solution. Stir with a glass rod and incubate for 1-3 hours at 37°. Carefully separate the fibrin from the walls of the tube by pouring cold saline on the top. Slowly rotate the rod to wind the fibrin around it. Press the rod occasionally against the walls of the tube to express moisture. Wash the fibrin with cold saline and add to a centrifuge tube containing 8 ml. of 3% sodium hydroxide solution. Heat for 5 minutes at 60° and add 0.3 ml. of 20% cupric sulfate pentahydrate solution. Stir for 20-30 seconds and centrifuge for 5 minutes. Read at once at 500 $m\mu$.

Precipitation as fibrin by calcium chloride, dissolved in trypsin and read in the ultraviolet. Plasma. Precipitate a 0.5-ml. sample with 0.5 ml. of 0.3% calcium chloride solution. Remove the clot, which contains the fibrin, with a glass rod. Wash several times in 0.9% sodium chloride solution. Transfer the rod and clot to a tube containing 1 ml. of a pH 9 buffer prepared by mixing 420 ml. of 1:150 ammonium hydroxide solution and 580 ml. of 0.535% ammonium chloride solution. Add 0.125 ml. of a solution containing 50 mg. of trypsin in 20 ml. of 0.0025 *N* hydrochloric acid. Incubate at 37° for 1 hour to dissolve the clot. Rinse the rod with the buffer and remove. Dilute to 5 ml. with the buffer and read at 280 $m\mu$.

Precipitation by ammonium sulfate. Biuret reagent. Plasma. To 1 ml. of citrated plasma, diluted 1:1 with 0.9% sodium chloride solution, slowly add 0.25 ml. of saturated ammonium sulfate solution and agitate the tube. Let stand at room temperature for 15 minutes. Centrifuge, and decant

the supernatant liquid. Drain the tube on filter paper for several minutes. Dissolve the precipitate in 2.5 ml. of 0.9% sodium chloride solution and develop a 2-ml. aliquot by the biuret method, using the potassium sodium tartrate biuret reagent (p. 359).

Turbidimetrically by 12% ammonium sulfate. Plasma. To prepare the ammonium sulfate solution, dissolve 133.33 grams of ammonium sulfate and 10 grams of sodium chloride in water. Add 0.025 gram of Merthiolate to suppress microbial growth. Dilute to 1 liter with water and adjust the pH to 7 with 40% sodium hydroxide solution, using 0.02% phenol red as an indicator. This gives a 12% ammonium sulfate solution when combined with the protein.

To a 0.5-ml. sample, add 0.5 ml. of 0.85% sodium chloride solution to prevent formation of large flocculating particles. Add 9 ml. of the ammonium sulfate solution and shake well. Read the turbidity at 450 m μ against a blank containing 0.5 ml. of plasma and 9.5 ml. of 1% sodium chloride solution. Optical densities less than 0.1 give an average error of 10% and should not be used.

By Octab, octadecyl dimethyl benzyl ammonium chloride. Plasma. To prepare the phosphate buffer at pH 5.1, add 2.3% sodium chloride solution to a 1.4% monobasic sodium phosphate solution to make the solution 0.4 M with respect to sodium chloride.

To a 1-ml. oxalated sample, add 4 ml. of 1% sodium chloride solution and 2 ml. of saturated ammonium sulfate solution. Mix thoroughly. After 5 to 10 minutes, centrifuge at 1500 rpm. for 10 minutes. The fibrinogen collects at the bottom as a pellet. Remove the supernatant liquid containing other proteins by gentle decantation. Remove any remaining supernatant liquid with a cotton gauze pledget. Dilute to 5 ml. with 1% sodium chloride solution and mix with a wooden applicator stick to dissolve the precipitate. To a 0.1-ml. aliquot add 8.9 ml. of the phosphate buffer and 1 ml. of 0.1% Octab solution. Mix immediately by inverting several times. Read at 440 m μ , 15 minutes after addition of the detergent.

GELATIN

Gelatin is determined by its hydroxyproline content. The sample is hydrolyzed to convert the protein to the amino acids, the hydroxyproline separated chromatographically from tyrosine and determined by standard

hydroxyproline techniques.¹³⁹ Tryptophan is destroyed during acid hydrolysis. Alternatively, the proline is separated from the hydroxyproline on Dowex 50, sodium salt, and the hydroxyproline developed with ninhydrin to form a red color.¹⁴⁰ This method is also applicable to oxypolygelatin.

The optimum hydrolysis time for gelatin is 1.5 hours at 145° with hydrochloric acid. Longer time results in loss of hydroxyproline. By this method, Beer's law is followed for 0.001-0.004 mg. of hydroxyproline. The biuret reaction is also applicable to gelatin determination.¹⁴¹

Procedure—*As hydroxyproline by ninhydrin. Plasma or urine.*¹⁴² To prepare a citrate buffer, mix 500 ml. of 5.8% sodium citrate at pH 5 and 110 ml. of 1:10 hydrochloric acid, 390 ml. water, and 5 ml. of thiodiglycol. Adjust to pH 3.42 with 4% sodium hydroxide solution.

Wash 1 pound of 250-500 mesh Dowex 50, hydrogen form, with 1:2 hydrochloric acid with very gentle suction, using 4-8 liters of acid or until the filtrate is nearly colorless. Wash with water twice, followed by washing with 8% sodium hydroxide solution until the filtrate is alkaline. Suspend the resulting sodium salt of the resin in 3 times its volume of 4% sodium hydroxide solution, and heat at 100° for 3 hours with occasional shaking. Let settle for 30 minutes and decant the supernatant fluid. Replace it with hot 4% sodium hydroxide solution and repeat the procedure 4 more times. Filter the resin, wash free of alkali, and pass through a 120-mesh screen with 6-8 liters of water.

The chromatographic column is 15 × 0.9 cm., fitted with a tap or other valve. Place a pad of fine glass wool at the bottom of the tube. Pour in a suspension of the purified Dowex 50, in the pH 3.42 citrate buffer. The height of the settled resin is 8 cm. Wash the resin with 50 ml. of buffer.

The suitable flow rate of 20 ml. per hour during addition of the hydrolyzate to the column is maintained by an aspirator system or nitrogen cylinder with suitable reduction valve to supply a pressure equivalent to a 3-ft. head of water. During development of the column, the flow rate is maintained with a reservoir of buffer.

Use a 3-ml. sample or a sample containing approximately 20 mg. of gelatin. Add an equal volume of concentrated hydrochloric acid and seal

¹³⁹ A. A. Leach, *Anal. Biochem.* **2**, 529-34 (1961).

¹⁴⁰ Charles J. Rogers, J. R. Kimmel, Maxine E. Hutchin and Harold A. Harper, *J. Biol. Chem.* **206**, 553-559 (1954).

¹⁴¹ R. Ardry and O. Risbec, *Ann. pharm. franc.* **12**, 428-36 (1954).

¹⁴² Stanford Moore and William H. Stein, *J. Biol. Chem.* **192**, 663-81 (1951).

with an oxygen flame. Heat in an oil bath at 145° for 1.5 hours. Cool, open the tube, and evaporate one-half of the contents to dryness to remove hydrochloric acid. Dissolve the dried residue in 5 ml. of citrate buffer. Filter the solution of hydrolyzate. Add 1-2 ml. of the hydrolyzate containing up to 0.2 mg. of hydroxyproline to the column. Immediately after the sample enters the resin, add 1 ml. of citrate buffer, dropwise, to wash all sample into the resin. Wash the column with six 5-ml. portions of buffer and collect the effluent in 2-ml. quantities. Adjust the pH of the effluent to 7 with a neutralizing mixture. To determine the quantity of sodium hydroxide required for this neutralizing mixture, titrate 25 ml. of citrate buffer to pH 7 with 8% sodium hydroxide solution. This amount of sodium hydroxide when brought up to 25 ml. with 0.1 *M* Sorensen phosphate buffer at pH 7 constitutes the neutralizing mixture.

To a 0.1-ml. aliquot of the 2 ml. effluent, add 0.1 ml. of neutralizing mixture. Add 2.5 ml. of benzene. Immerse the tube with a bulb in a water bath at 75° and attach it to a vibrator. Start the vibrator and add 0.2 ml. of 1.7% ninhydrin solution in methyl Cellosolve. After 15 minutes, remove the tube, and let cool in the dark. Decant the benzene layer through benzene-washed cotton. Wash the tube with several small portions of benzene. Add the washings to the solution and dilute to 5 ml. with benzene. If the extract is turbid, add a few crystals of anhydrous sodium sulfate. Read at $570\text{ m}\mu$ against a reagent blank prepared with 0.1 ml. citrate buffer in place of the sample.

Other methods for hydroxyproline, page 299, are also applicable to the filtered hydrolyzate.

By biuret. Plasma. To prepare the copper-sodium reagent, dissolve 1.5 grams of cupric sulfate pentahydrate and 120 grams of sodium hydroxide in water, and dilute to 500 ml.

Mix a 0.1-ml. sample with 3 ml. of 0.9% sodium chloride solution. Add an equal volume of biuret reagent and read after 15 minutes at $535\text{ m}\mu$. Mix a 0.2-ml. sample with 6 ml. of 2.5% trichloroacetic acid solution and centrifuge. Mix the supernatant liquid with an equal volume of biuret reagent and read at $535\text{ m}\mu$. Dilute the biuret reagent 1:1 with 0.9% sodium chloride solution and read at $535\text{ m}\mu$.

OXYPOLYGELATIN

Trichloroacetic acid precipitates gelatin from other proteins and nitrogenous compounds. Addition of sulfuric acid to the system separates

the oxypolygelatin.¹⁴³ The maximum turbidity is achieved with a final trichloroacetic acid concentration at a minimum of 5.34%. Higher concentrations of trichloroacetic acid favor precipitation of the gelatin only. The turbidity remains constant for 10 to 15 minutes after an initial 10-minute interval. The procedure is applicable to 50-500 mg. of oxypolygelatin per 100 ml.

Procedure—Plasma. Add 0.05 ml. of heparin per 10 ml. of sample. Dilute a 1-ml. heparinized sample with 17 ml. of water. For samples over 500 mg. of oxypolygelatin per 100 ml., use a 0.5-ml. sample diluted with 17.5 ml. of water. Add 2 ml. of 50% trichloroacetic acid solution. Mix and centrifuge. To 5 ml. of the supernatant liquid, add 1 ml. of concentrated sulfuric acid. Shake, and let stand for 10 minutes. Tap to remove air bubbles. Read against a blank with a blue filter.

Urine. Dilute a 1-ml. sample to 4.5 ml. with water. Add 0.5 ml. of 50% trichloroacetic acid solution and mix. Add 1 ml. of concentrated sulfuric acid and mix. After 10 minutes, tap to remove air bubbles and read against a blank. To prepare the blank, omit the trichloroacetic acid and add the sulfuric acid to an equivalent concentration of urine to eliminate the interference of urine color.

PEPTONE

Peptone is determined by phosphotungstic-phosphomolybdic acid.¹⁴⁴

Procedure—To 1 ml. of sample solution to the range of 1-3 mg. per ml., add 7.5 ml. of water, 0.5 ml. of 4% sodium hydroxide solution, and 0.3 ml. of the reagent (Vol. III, p. 116). Mix, and read after exactly 20 minutes at 580 m μ against a reagent blank.

EGG PROTEIN

The violet color of the protein in egg is developed by the biuret reaction with copper sulfate and potassium hydroxide.¹⁴⁵

¹⁴³ Charles J. Rogers, *Am. J. Clin. Pathol.* **23**, 638-44 (1953); T. I. Pristoupil, V. Tomanova, and J. Nikl. *Chem. Listy* **50**, 386-7 (1956).

¹⁴⁴ Pierre Mesnard and Guy Devaux, *Bull. soc. pharm. Bordeaux* **99**, 73-6 (1960).

¹⁴⁵ Amina Sinigaglia, *Boll. lab. chim. provinciali* **6**, 47-8 (1955).

Procedure—Pasta. Shake a 10-gram sample with 100 ml. of water for 2 minutes. Filter after 1 hour. To the clear filtrate, add 1 ml. of 10% copper sulfate solution and 1 ml. of 10% potassium hydroxide solution. Read the violet color at 550 m μ .

CASEIN

The common method for casein in milk samples employs the biuret reaction¹⁴⁶ (Vol. IV, p. 194).

Procedure—The biuret reagent for this purpose contains 1.5 grams of copper sulfate pentahydrate, 6 grams of potassium sodium tartrate, 30 grams of sodium hydroxide, and 1 gram of potassium iodide per liter.

Dilute a 1-ml. sample of milk with 4 ml. of water. Mix a 3-ml. aliquot with 0.15 ml. of 1:10 acetic acid. Centrifuge the casein and wash twice with 10-ml. portions of 95% ethanol. Wash twice with 10-ml. portions of ethyl ether and dissolve in 1 ml. of 4% sodium hydroxide solution. Dilute to 5 ml. Add 4 ml. of the reagent to a 1-ml. aliquot of the sample. Centrifuge and read at 430 m μ .

Alternatively, dilute a 0.1-ml. sample with 0.5 ml. of water and shake with 6 ml. of a 1:1 alcohol-acetone mixture. Heat for 5 minutes at 72-75° and centrifuge. Decant the supernatant liquid and shake the residue vigorously with 5 ml. of the biuret reagent. Read after 10 minutes, using 3% casein solution as the standard.

ZEIN

Zein, a corn protein, can be precipitated from an ethanol solution by addition of water for turbidimetric or nephelometric reading. When sodium chloride solution is the precipitant, the reproducibility of the method improves but the stability decreases.¹⁴⁷ Vigorous shaking causes the suspended particles to aggregate into larger stringy particles, thus adversely affecting results. The method is also dependent upon the concentration of ethanol. The turbidity of zein solutions in concentrations of 0.1 to 1 mg. is read by comparison. For greater sensitivity at lower ranges of 0 to 0.04 mg., the turbidity is measured with a nephelometer. Beer's law is followed from 0.1-0.5 mg.

¹⁴⁶ C. Pandolfi, *Boll. soc. ital. biol. spec.* **29**, 1207-8 (1953); M. Leclerc and A. Khodabandeh, *Ann. pharm. franc.* **13**, 749-52 (1955).

¹⁴⁷ E. M. Crane, Carol A. Jones and Joyce A. Boundy, *Cereal Chem.* **34**, 456-62 (1957).

Procedure—*Samples containing 0.1-1 mg. of zein.* Using a pipet, blow 6 ml. of 1% sodium chloride solution into a 2-ml. sample in 70% ethanol. Remove small bubbles on the tube surface by rotating the tube at an angle. Read after 1 hour at 590 m μ .

Samples containing up to 0.04 mg. zein. Follow the procedure for samples containing 0.1-1 mg. zein using a 1-ml. sample and reading with a nephelometer after 10 minutes.

ELASTIN

Elastin is treated with orcein, and unreacted orcein is removed with acid ethanol. Elastin is then hydrolyzed with pancreatin and the stain thus brought into solution is read at 590 m μ ¹⁴⁸ (cf. Vol. IV, p. 196).

Sample—*Tissue.* Prepare acid ethanol containing 0.1 ml. of concentrated hydrochloric acid per 100 ml. of 95% ethanol. As orcein reagent, prepare a stock solution containing 1 gram of orcein, 100 ml. of anhydrous ethanol, and 1 ml. of concentrated hydrochloric acid. Before use, dilute 1 ml. of stock solution with 9 ml. of acid ethanol.

Finely mince a 1.5-gram sample to 2 mm. particles and wash with water to remove blood. Extract at 40° for 8 hours with three 100-ml. portions of acetone. Dry at 70° for 12 hours. Grind the residue and macerate a 30-50 mg. sample with 10 ml. of 0.4% sodium hydroxide solution for 8 hours at 25° with frequent mixing. Centrifuge, and suspend the residue twice in 10 ml. of acid ethanol at 38° for 30 minutes. Centrifuge, and treat with 20 ml. of orcein reagent. The staining is completed in 24 hours at 25° with occasional stirring. Centrifuge, and wash the residue with 20-ml. portions of acid ethanol until the free color is removed. The usual wash periods are 2 hours for the first, 12 for the second, and 5 for the last. Remove ethanol by centrifuging and wash the residue with 10 ml. of glycocoll-sodium chloride-sodium hydroxide solution at pH 9.5 at 38° for 1 hour. Replace this buffer by 5 ml. of fresh buffer.

Prepare 4% pancreatin solution in glycocoll-sodium chloride-sodium hydroxide buffer at pH 9.5, which has been filtered before use. Digest the elastin with 5 ml. of pancreatin reagent at 38° for 1 hour with frequent stirring. Centrifuge out all undigested material. A slight turbidity from the pancreatin solution does not interfere. Read at 590 m μ against a blank containing 5 ml. of buffer and 5 ml. of pancreatin solution.

¹⁴⁸ V. Scarselli, *Giorn. biochim.* **7**, 20-7 (1958); *Nature* **184**, 1563 (1959).

CHAPTER 5

AROMATIC PRIMARY, SECONDARY, AND TERTIARY AMINES AND AMIDES

MANY METHODS for nitrated cyclic amino compounds appear in Chapter 1 of this volume. They include *nitrofuramine* (p. 31), *2,6-dichloro-4-nitroaniline* (p. 24), nitro- and nitrosodiphenylamines (p. 44), dinitrophenylamino groups (p. 50), 3,5-dinitrobenzamide (p. 48), and dichloran (p. 24). For determination of *N*-methylaniline by pyrocatechol, see dibutylamine, page 137. Table 21 gives significant fluorescent reactions applicable to these classes of compounds.¹

TABLE 21. SIGNIFICANT FLUORESCENT REACTIONS FOR AROMATIC PRIMARY, SECONDARY, AND TERTIARY AMINES AND AMIDES

Compound	pH	Excitation wave length m μ	Emission wave length m μ
Lysergic acid diethanolamide	7	325	465
Bromolysergic acid	1	315	460
Procaine	11	275	345
Procaine amide	11	295	385
Thiamylol (Surital)	13	310	530

Aromatic amines and *chloranil*, 2,3,5,6-tetrachloro-1,4-benzoquinone form adducts in chloroform.² This qualitative reaction has quantitative applicability according to details given in Table 22.

Methods for aniline are in general applicable to *toluidines* (cf. Vol. IV, p. 225). Thus they are diazotized and coupled with *N*-ethyl-1-naphthylamine.³ The reaction of *p*-toluidine with ferric ion to give a brown-red color is applicable.⁴

Microquantities of *aniline*, *methylaniline*, and *dimethylaniline* are de-

¹ Sidney Udenfriend, *et al.*, *J. Pharmacol. Exptl. Therap.* **120**, 26-32 (1957).

² O. Freliden and L. Goldschmidt, *Mikrochim. Acta* **1**, 338 (1937).

³ V. Kratochvil, *Z. anal. Chem.* **183**, 267-72 (1961).

⁴ V. A. Vinogradova, *Gigiena i Sanit.* **No. 11**, 48 (1954).

TABLE 22. LIGHT ABSORPTION DATA OF ADDUCTS BETWEEN AROMATIC AMINES AND CHLORANIL, IN CHLOROFORM, AT 20°

<i>Amine</i>	<i>Color</i>	$\lambda_{\max}(m\mu)$
Aniline	Violet	542
α -Naphthylamine	Blue	645
β -Naphthylamine	Blue	635
3-Phenanthrylamine	Green	652
9-Phenanthrylamine	Brown	487
1-Aminotriphenylene	Black	No maximum
2-Aminotriphenylene	Black	No maximum
N-Methylaniline	Blue	602
N-Ethylaniline	Blue	605
N-Dimethylaniline	Blue	660
N-Diethylaniline	Green	765
Diphenylamine	Blue	647
Triphenylamine	Blue	660
<i>o</i> -Chloroaniline	Red-brown	512
<i>m</i> -Chloroaniline	Red-brown	497
<i>p</i> -Chloroaniline	Violet	520
<i>o</i> -Bromoaniline	Red-brown	515
<i>m</i> -Bromoaniline	Red-brown	502
<i>p</i> -Bromoaniline	Violet	535
<i>o</i> -Toluidine	Lilac	562
<i>m</i> -Toluidine	Lilac	550
<i>p</i> -Toluidine	Blue	580
<i>o</i> -Anisidine	Blue	586
<i>m</i> -Anisidine	Blue	575
<i>p</i> -Anisidine	Green	600
<i>o</i> -Phenylene diamine	Green	— ^a
<i>m</i> -Phenylene diamine	Brown	— ^a
<i>p</i> -Phenylene diamine	Green	— ^a
Anthranilic acid ^b	Red-brown	515

^a Solution goes rapidly opaque.^b Solvent: acetone.

terminated in mixtures such as those absorbed from air in 1% ethanol.⁵ The aniline is diazotized and coupled with 1-naphthol. The methylamine is oxidized with Chloramine-B, and the oxidation product coupled with phenol in alkaline solution for reading at 619 $m\mu$. The dimethylaniline is diazotized and coupled with 1-naphthol. The dimethylamine is diazotized and coupled with *m*-nitroaniline in 20% acetic acid.

Mixtures of *salicylamide* and *p*-aminobenzoic acid, are read at pH

⁵ A. A. Belyakov and N. V. Gorbyleva, *Zhur. Anal. Khim.* **12**, 545-9 (1957).

10 without separation.⁶ The same is true of salicylamide and caffeine, with or without phenacetin present. *Salicylamide* and *dimethyl-p-phenylenediamine* buffered to pH 8.4 are oxidized by sodium hypochlorite to indophenol.⁷ The color body is extracted with isobutanol for reading at 650 m μ . *p-Acetamidosalicylic acid* and *p-acetaminosalicylic acid* are urinary metabolites of *p-aminosalicylic acid*. They are discussed briefly under the latter, page 387). For separation of 3-hydroxyanthranilic acid from tryptophan metabolites for development with *p*-dimethylaminobenzaldehyde, see kynurenine, on page 293. *Anthranilic acid* can be determined by *p*-dimethylaminobenzaldehyde in the presence of tryptophane and indole (p. 267).

o-Toluidine is determined by tetrazotizing and coupling with *N*-ethyl-1-naphthylamine.⁸ *Benzidine*, *diphenylene* and *o-dianisidine* give the same reaction. Therefore, the reaction is also applicable to them (p. 377). *Dibenamine*, *N*-(2-chloroethyl) *dibenzylamine*, and the corresponding alcohol are determined by reaction with indicators,⁹ a reaction shown for benadryl in Volume IV, page 72. The use of bromocresol purple in place of methyl orange increases the sensitivity by a factor of 2 to 5.

p-Aminobenzoic acid can be read at pH 10 without separation from caffeine and phenacetic.¹⁰ The same is true of salicylamide. *Aminohippuric acid* and *aminobenzoic acid* in urine can be separated on a column of Dowex 50-X-12.¹¹ Each is determinable by reaction with *N*-(1-naphthyl) ethylenediamine. The *o*- and *p*-isomers of each are determinable by the rate of reaction and, therefore, of color development.

When *antipyrine* is diazotized, it couples in acetic acid solution with 4,5,1-aminonaphtholsulfonic acid.¹² The color is read at 565 m μ . The recovery from plasma is nearly 10% low. *Ferric anthranilate* can be extracted into pentanol and the ferric ion determined at pH 3.7-5.0. The maximum is at 465-475 m μ .¹³ *o-Phenylenediamine* is determined in the presence of the *m*- and *p*-isomers by reacting with selenious acid at pH 1-2 to form benzo-2,1,3-selenadiazole.¹⁴ The absorption is at 335 m μ .

⁶ Włodzimierz Żyżvski, *Acta Polon. Pharm.* **17**, 277-86 (1960).

⁷ Konosuke Murai, *Yakuzaigaku* **21**, 58-60 (1961).

⁸ V. Kratochvil, M. Matrká and J. Marhold, *Collection Czech. Chem. Commun.* **25**, 101-7 (1960).

⁹ K. H. Boltze, D. Weyland and H. Hofmann, *Biochem. Z.* **327**, 239-44 (1955).

¹⁰ Włodzimierz Żyżvski, *Acta Polon. Pharm.* **17**, 277-86 (1960).

¹¹ S. L. Tompsett, *Clin. Chim. Acta* **8**, 308-11 (1963).

¹² Toshio Nambara, *Yakugaku Zasshi* **80**, 460-4 (1960).

¹³ D. L. Dinsel, *Dissert. Abstr.* **24**, 51 (1963).

¹⁴ L. Barcza, *Acta chim. hung.* **41**, 91-5 (1964).

m-Diethylaminophenol gives a brown color as the nitroso derivative.¹⁵ To develop 5 ml. of about 1.65% solution of the test substance in 1:120 hydrochloric acid, add 2 ml. of 7% sodium nitrite solution. After 20 minutes at 25°, neutralize with 20% potassium hydroxide solution and dilute to 100 ml. Read with or without further dilution. 4-Aminosalicylic acid is determined in blood or tissue extract by its color with ferric chloride.¹⁶ Any *N*-acetyl derivatives of the subject compound must first be extracted with ethyl acetate. The color is read at 540 m μ . It is also determined by dimethylaminobenzaldehyde.¹⁷

Di-isopropylamine dichloroacetate is nitrosated in acetic acid at 40-60°. The resulting nitrosodi-isopropylamine is extracted from potassium hydroxide solution with 5 ml. of cyclohexane for reading at 235 m μ .¹⁸ About 0.2 mg. of the test substance in 4 ml. of 50% acetic acid is completely nitrosated with 2 ml. of 10% sodium nitrite solution within 5 minutes. *Neostigmine bromide* is hydrolyzed in alkaline solution to give *m*-dimethylaminophenol.¹⁹ The latter is then nitrated and read. *Chlor-diazepoxide* is hydrolyzed by concentrated hydrochloric acid at 100° to give 2-amino-5-chlorobenzophenone. This can be read after diazotizing and coupling with 2-naphthol in alkaline solution.²⁰

Acridine is a mixture of 2,8-diamino-10-methylacridinium chloride and 2,8-diaminoacridine. Buffered at pH 6.0, it is read fluorimetrically to 0.01 mg. %.²¹ Tormentil does not interfere. It is also diazotized and coupled with *N*-(1-naphthyl)ethylenediamine for reading.²² Flavines are separated by paper chromatography, converted to lumiflavines by photochemical decomposition, and read.²³

Various rubber antioxidants are extractable and determinable.²⁴ Thus *N*-phenyl-1-naphthylamine extracted with acetone gives a maximum at

¹⁵ L. I. Pykhtina, *Sb. Nauch. Trud. Permsk. Politekh. Inst.* **14**, 86-8 (1963).

¹⁶ R. Fried, *Arch. Pharm., Berlin* **295**, No. 9 (1962); *Mitt. dtsh. pharm. Ges.* **32**, 157-9 (1962).

¹⁷ Michel Brous, *Am. J. Clin. Pathol.* **26**, 552 (1956); O. Quadrat, *Časopis lékařů českých* **99**, 745-9 (1960).

¹⁸ Masao Maruyama and Kazue Hasegawa, *Japan Analyst* **10** (5), 518-22 (1961).

¹⁹ I. Solomon-Ionescu, M. Prodescu, and F. Ciciureanu, *Farmacii* (Bucharest) **10**, 551-3 (1962).

²⁰ J. Bäumler and S. Rippstein, *Helv. Chim. Acta* **44**, 2208-10 (1961).

²¹ T. Bičan-Fišter, *Acta Pharm. Jugoslav.* **10**, 161-6 (1960).

²² František Sokol, *Chem. Zvesti* **9**, 489-93 (1955).

²³ Kunio Yagi, Hiroshi Kondo, and Jun Okuda, *J. Biochem. (Tokyo)* **51**, 231-2 (1962).

²⁴ Isao Yamaji, Masako Sawada and Takao Yamashina, *J. Soc. Rubber Ind. Japan* **35**, 774-8 (1962).

575 $m\mu$. *N*-phenyl-2-naphthylamine gives a maximum at 500 $m\mu$. *N,N'*-Di-2-naphthyl-*p*-phenylenediamine treated with benzoyl peroxide gives a maximum at 480 $m\mu$. Similarly treated, *N,N'*-diphenyl-*p*-phenylenediamine has a maximum at 450 $m\mu$, and *N*-cyclohexyl-*N'*-phenyl-*p*-phenylenediamine, at 420 $m\mu$.

Anthraceneamine is determined by heating for 6 minutes with 4-azobenzenediazonium fluoborate followed by cooling and addition of hydrochloric acid.²⁵ *Phenylnaphthylamine* is read fluorescently at 430 $m\mu$.²⁶

To determine 5-substituted 2,4-disulfamoylaniline derivatives,²⁷ boil a 5-ml. sample of the solution containing 0.085-0.175 mg. in 4% sodium hydroxide for 3 hours. Cool, and add 15 ml. of 1:10 hydrochloric acid, 1 ml. of 7% sodium nitrite solution. After a few minutes, add 1 ml. of 4% ammonium sulfamate solution and 0.5 ml. of 0.5% *N*-(1-naphthyl)-ethylenediamine hydrochloride. Dilute to 50 ml. and read at 515 $m\mu$ after 15 minutes against a blank.

For determination of *neopyrithiamine* in the presence of thiamine, the latter is destroyed with alkali. Thereafter, the thiochrome method is applied as for thiamine.²⁸ *Pyrithiamine* is determined by the thiochrome method (p. 435). *1-Naphthal conjugates* are determined by the method for *Sevin* (p. 437).

AROMATIC AMINES

Aromatic amines, phenols, and aldehydes as phenylhydrazones are determined by the formation of colored compounds with maxima at 380-590 $m\mu$ when coupled with the diazo reagent, 3-phenyl-5-nitrosamine-1,2,4-thiadiazole.²⁹ The following aromatic amines: aniline, isomeric chloroanilines, toluidines, and xyloidines, sulfanilic, and metanilic acids are coupled with the following coupling agents after diazotization: 3-hydroxy-2-naphthoic acid, 2-naphthol-3,6-disulfonic acid (R-salt), *N*-sulfatoethyl-*m*-toluidine, *N*-1-naphthylethylenediamine and *N'*-diethyl-*N*-1-naphthyl-propylenediamine.³⁰ *N*-1-naphthylethylenediamine, *N'*-diethyl *N*-1-

²⁵ Eugene Sawicki, James Noc and Francis T. Fox, *Talanta* **8**, 257-64 (1961).

²⁶ Kunio Yagi, Toshikazu Tabata, Etsu Kotaki and Takao Arakawa, *Vitamins (Japan)* **9**, 391-2 (1955).

²⁷ M. Glielardoni and M. Fedi, *Boll. Chim. Farm.* **101**, 26-30 (1962).

²⁸ G. Rindi and V. Perri, *Boll. Soc. Ital. Biol. Sper.* **35**, 2025-8 (1959).

²⁹ Maurice Pesch, Jaroslav Bartos and Jean F. Burtin, *Talanta* **5**, 213-5 (1960).

³⁰ John W. Daniel, *Analyst* **86**, 640-3 (1961).

naphthylpropylenediamine and *N*-sulfatoethyl-*m*-toluidine give the same order of sensitivity and are much superior to 2-naphthol-3,6-disulfonic acid or 3-hydroxy-2-naphthoic acid.

After reaction with an excess of mercuric acetate in acetic acid, arylamines are developed with ammonium rhodanide, which produces a turbid solution with the mercury compound.³¹ Addition of 0.5 ml. of 1:1 acetic acid after addition of the rhodanide reduces the interference of tertiary amines. Secondary aromatic amines react with carbon disulfide to form dithiocarbamic acid, which reacts with cupric ion to form a yellow salt.³² See secondary aliphatic amines, page 79, for details.

A basic reaction of aromatic amines with aromatic amines is that with diazotized *p*-nitroaniline.³³ It is applied to *N*-phenyl-1-naphthylamine (p. 423).

Aromatic amines are converted to their azo derivatives. Then they are chromatographed on magnesium oxide containing 10-15% of magnesium hydroxide.³⁴ This will separate 5-10 microgram amounts of aniline and *o*-, *m*-, and *p*-toluidines; *o*-, *m*-, and *p*-anisidines; aniline and diphenylamine; and 1- and 2-naphthylamines. The original must be consulted for eluents.

Phenitrazole is dehydrated in acid solution to a diazonium compound that will couple with arylamines, phenols, and aldehydes to form the phenylhydrazone.³⁵ Aniline, 1- and 2-naphthylamines, 1- and 2-anthramines, and their *N*-alkyl and *N,N*-dialkyl derivatives are determinable with azobenzene-4-diazonium fluoborate.³⁶ There is interference by diphenylamine and its derivatives, carbazole, and indole.

Diphenylpicrylhydrazyl is an intensely violet free radical. It reacts with amines by abstracting a hydrogen atom to form yellow diphenylpicrylhydrazine. The decrease in violet color is used for the determination.³⁷ The rate of reaction differs for each amine. Therefore, the standard curve must be prepared with the specific amine being determined. Also, on occasion, this may permit determination of one amine in the presence of another. Table 23 shows the relative reactivity of many aromatic and

³¹ A. A. Belyakov, *Nauch. Raboty Khim. Lab. Gor'kovsk. Nauch.-Issledovatel. Inst. Gigeny Truda i Professional Boleznei Sbornik* 1957, No. 6, 61-3.

³² Gerald R. Umbreit, *Anal. Chem.* 33, 1572-3 (1961).

³³ W. S. Levine and W. A. Marshall, *Anal. Chem.* 27, 1019-22 (1955).

³⁴ L. Nebbia and B. Pagani, *Chim. e Ind.* 41, 872-4 (1961).

³⁵ Maurice Pesetz, Jaroslav Bartos and Jean F. Burtin, *Talanta* 5, 213-15 (1960).

³⁶ E. Sawicki, J. I. Noe and F. T. Fox, *Talanta* 8, 257-64 (1961).

³⁷ G. J. Papariello and M. A. M. Janish, *Anal. Chem.* 37, 899-902 (1965).

TABLE 23. RELATIVE REACTIVITY OF VARIOUS AMINES WITH DIPHENYLPICRYLHYDRAZYL AT 60°^a

<i>Compound</i>	<i>Amine: reagent ratio</i>	<i>Reactivity</i>
Primary aromatic amines		
Aniline	10:1	Good
<i>m</i> -Nitroaniline	100:1	Poor
<i>p</i> -Nitroaniline	100:1	Fair
<i>p</i> -Toluidine	10:1	Good
<i>m</i> -Toluidine	10:1	Good
<i>o</i> -Toluidine	10:1	Good
<i>p</i> -Chloroaniline	100:1	Good
<i>p</i> -Chloroaniline	10:1	Fair
<i>m</i> -Chloroaniline	100:1	Good
<i>m</i> -Chloroaniline	10:1	Poor
<i>p</i> -Aminobenzoic acid	100:1	Good
<i>p</i> -Anisidine	1:1	Good
Secondary aromatic amines		
Methylaniline	1:1	Good
<i>N</i> -methyl- <i>p</i> -nitroaniline	100:1	No reaction
<i>N</i> -methyl- <i>p</i> -toluidine	1:1	Good
<i>N</i> -methyl- <i>m</i> -toluidine	1:1	Good
Tertiary aromatic amines		
Dimethylaniline	1:1	Good
Diethylaniline	1:1	Good
<i>N,N</i> -dimethyl- <i>p</i> -toluidine (25°)	1:1	Good
<i>N,N</i> -dimethyl- <i>m</i> -toluidine (25°)	1:1	Good
Primary aliphatic amines		
Isobutylamine	100:1	No reaction
<i>sec</i> -Butylamine	100:1	No reaction
<i>tert</i> -Butylamine	100:1	No reaction
<i>n</i> -Butylamine	100:1	No reaction
Hexylamine	100:1	No reaction
Benzylamine	100:1	Poor
Secondary aliphatic amines		
Diethylamine	100:1	No reaction
Methylbenzylamine	100:1	Fair
<i>N</i> -methylcyclooctylamine (25°)	100:1	Good
Tertiary aliphatic amines		
Tributylamine	100:1	Good
<i>N</i> -benzyldimethylamine	100:1	Good
Ethylidicyclohexylamine	100:1	No reaction

^a Evaluation of the reactivity of an amine with the reagent is based on the per cent of discoloration caused by the amine during a 30-minute reaction period using the indicated mole ratio: no reaction, 0-5% discoloration; poor, 5-10% discoloration; fair, 10-30% discoloration; good, 30-100% discoloration.

aliphatic amines. For total amines in water, see page 67. For a general reaction of 1,2-naphthoquinone-4-sulfonate see its application to procaine, page 407.

Procedure—*By 2-naphthol-3,6-disulfonic acid.* To prepare the reagent, dissolve 15-16 grams of the disodium salt of 2-naphthol-3,6-disulfonic acid in 500 ml. of water by heating, and make the solution just alkaline to Brilliant Yellow paper with 10.6% sodium carbonate solution. Cool, filter, and dilute to 1 liter with water.

Dissolve a 0.1-gram sample of the amine in 1:10 hydrochloric acid and dilute to 100 ml. with water. To a 1-ml. aliquot containing 0.01 mg. of amine, add 1 ml. of 1:5 hydrochloric acid and 1 ml. of 0.25% sodium nitrite solution. Stopper, and mix by inversion. Let stand at room temperature for 15 minutes.

To a portion of the diazotized solution, add 10 ml. of 10.6% sodium carbonate solution and 0.5 ml. of the reagent. Dilute to 25 ml. with water. Let stand according to the time specified in Table 24, and read at the wave length indicated in the table.

By N-sulfatoethyl-m-toluidine. Follow the procedure for 2-naphthol-3,6-disulfonic acid, ending with "Let stand at room temperature for 15 minutes."

To a portion of the diazotized amine, add 2 ml. of 25% sodium acetate solution and 2 ml. of 1% *N*-sulfatoethyl-*m*-toluidine solution. Add 0.5 ml. of concentrated hydrochloric acid and dilute to 10 ml. with water. For coupling periods in excess of 1 hour, add 1 ml. of 2.5% ammonium sulfamate solution before addition of sodium acetate. Develop according to the time indicated in Table 24.

By 3-hydroxy-2-naphthoic acid. Follow the procedure for 2-naphthol-3,6-disulfonic acid, ending with "Let stand at room temperature for 15 minutes."

To a portion of the diazotized amine, add 1 ml. of 2.5% ammonium sulfamate solution. After one minute, add 1 ml. of 0.6% 3-hydroxy-2-naphthoic acid solution in 10.6% sodium carbonate solution. Acidify with 1:1 sulfuric acid, using Congo red paper. Extract with five 5-ml. portions of chloroform and dilute the combined extracts to 25 ml. with chloroform. Develop according to time indicated in Table 24.

By N-1-naphthylethylenediamine or N'-diethyl N-1-naphthylpropyl-enediamine. Follow the procedure for 2-naphthol-3,6-disulfonic acid, ending with "Let stand at room temperature for 15 minutes."

TABLE 24. DETAILS OF COLORS PRODUCED WITH VARIOUS AMINES

Amine	Color formed with N'-naphthylthylamine				Color formed with N'-diethyl N-1-naphthylpropylenediamine				Color formed with N-sulfoethyl-m-toluidine			
	Optical density $\times 10^3$	Wavelength of maximum absorption $m\mu$	Color-development period, minutes	Optical density $\times 10^3$	Wavelength of maximum absorption $m\mu$	Color-development period, minutes	Optical density $\times 10^3$	Wavelength of maximum absorption $m\mu$	Optical density $\times 10^3$	Wavelength of maximum absorption $m\mu$	Color-development period, minutes	Optical density $\times 10^3$
Aniline	44	545	15	45	540	15	32	500	32	500	10	—
<i>o</i> -Toluidine	25	540	30	34	535	30	13	505	13	505	60	—
<i>m</i> -Toluidine	33	545	15	34	540	15	27	505	27	505	10	—
<i>p</i> -Toluidine	38	545	30	43	545	30	20	505	20	505	10	—
2,6-Xyldine	<1	530	60	1	545	60	<1	505	<1	505	60	—
2,4-Xyldine	13	545	120	18	540	120	18	505	18	505	180	—
2,3-Xyldine	4	535	60	8	530	60	2	515	2	515	60	—
3,4-Xyldine	22	550	30	22	555	30	40	515	40	515	45	—
2,5-Xyldine	5	535	60	8	535	60	11	500	11	500	120	—
<i>o</i> -Chloroaniline	30	540	15	30	535	15	30	500	30	500	60	—
<i>m</i> -Chloroaniline	35	540	15	35	535	15	33	500	33	500	10	—
<i>p</i> -Chloroaniline	36	545	15	37	540	15	33	505	33	505	10	—
Sulfanilic acid	27	535	15	27	530	15	27	505	27	505	15	—
Metamlic acid	25	535	15	24	535	15	—	—	—	—	—	—

Amine	Color formed with 2-naphthol-3,6-disulfonic acid				Color formed with 3-hydroxy-2-naphthoic acid			
	Optical density $\times 10^3$	Wavelength of maximum absorption $m\mu$	Color-development period, minutes	Optical density $\times 10^3$	Wavelength of maximum absorption $m\mu$	Color-development period, minutes	Optical density $\times 10^3$	Wavelength of maximum absorption $m\mu$
Aniline	32	500	30	6	485	30	6	485
<i>o</i> -Toluidine	2	505	30	11	485	60	11	485
<i>m</i> -Toluidine	5	505	30	9	485	30	9	485
<i>p</i> -Toluidine	9	505	30	9	495	30	9	495
2,6-Xyldine	<1	500	60	4	485	60	4	485
2,4-Xyldine	5	515	60	7	495	60	7	495
2,3-Xyldine	1	510	60	3	485	60	3	485
3,4-Xyldine	7	515	30	4	510	30	4	510
2,5-Xyldine	3	505	60	7	485	120	7	485
<i>o</i> -Chloroaniline	5	500	30	4	485	15	4	485
<i>m</i> -Chloroaniline	6	500	30	4	485	15	4	485
<i>p</i> -Chloroaniline	6	500	30	5	485	15	5	485

To a portion of the diazotized amine, add 1 ml. of 2.5% ammonium sulfamate solution. After 1 minute, add 1 ml. of 1% reagent solution and dilute to 10 ml. with water. Alternatively, to a portion of the diazotized amine, add 1 ml. of 2.5% ammonium sulfamate solution. After 1 minute, add 2 ml. of 25% sodium acetate solution and 1 ml. of 1% reagent solution. Add 0.5 ml. of concentrated hydrochloric acid to develop the color and dilute to 10 ml. with water. Develop according to the time indicated in Table 24.

By 3-phenyl-5-nitroamino-1,2,4-thiadiazole. To 1-ml. of an ethanol solution of the sample, add 1 ml. of 0.4% 3-phenyl-5-nitroamino-1,2,4-thiadiazole solution in ethanol and 1 ml. of 65% perchloric acid. Let stand for 5 minutes. Cool in ice and add 3 ml. of ice-cold 20% sodium hydroxide solution. Read after 5 minutes.

By turbidity with ammonium rhodanide. To a 5-ml. sample of arylamine solution containing 0.005-0.1 mg. of amine in 0.1% acetic acid, add 0.2 ml. of filtered mercuric acetate solution containing 20 mg. per ml. in 0.5% acetic acid. Heat for 10 minutes at 85-100°. Cool, and add 0.1 ml. of 10% ammonium rhodanide solution. Read the turbidity. The milky white turbidity lasts for 8-10 hours.

As dithiocarbamic acid. See secondary aliphatic amines, page 79.

By phenitrazole. Arylamines and phenols. To 1 ml. of ethanolic sample, add 1 ml. of 0.4% solution of 5-nitrosamino-3-phenyl-1,2,4-thiadiazole (phenitrazole). Add 1 ml. of 65% perchloric acid. Let the mixture stand for 5 minutes. Chill in ice and add 3 ml. of prechilled 20% sodium hydroxide. Read after 5 minutes at room temperature at 380-590 $m\mu$.

By diphenylpicrylhydrazyl. In a stoppered tube, take 4 ml. of sample solution in methanol, 1 ml. of methanol, 1 ml. of *M* acetate buffer adjusted to pH 5, and 4 ml. of 2×10^{-4} micromolar solution of the reagent in methanol, prepared daily. After 15-60 minutes at room temperature or 60°, depending on the reactivity of the amine, read against a reagent blank at 515 $m\mu$.

By azobenzene-4-diazonium fluoborate. Mix a solution of the amine in methyl Cellosolve with a 0.1% solution of azobenzene-4-diazonium fluoroborate. Heat to 100° and make acid with concentrated hydrochloric acid. Read at 610-675 $m\mu$.

ANILINE

The diazo compound of aniline is coupled with 1-amino-8-naphthol-3,6-disulfonic acid to give a red dye³⁸ (cf. Vol. IV, p. 197). In acid copper-plating baths, copper is removed by addition of aluminum, which, in turn, is kept in solution with citrate. The pH is kept at 8.2-8.3 with sodium bicarbonate. The standards must be prepared with the plating bath. The diazo compound may also be coupled with 1-amino-8-naphthol-3,6-disulfonic acid (H-acid). The color does not follow Beer's law, but the results are within $\pm 4\%$.³⁹

Phenol,⁴⁰ *p*-phenylenediamine,⁴¹ and *N*-ethyl-1-naphthylamine⁴² are also reagents for aniline. The latter is read at 538 m μ .

Neither oxidation with hypochlorite (Vol. IV, p. 199) nor the corresponding hypobromite are specific for determination in air in the presence of phenol.⁴³ It is preferable to diazotize in dilute sulfuric acid and, after converting to an alkaline solution, couple with resorcinol. After 15 minutes, a distinct color is given by 0.0002 mg. For determination in air, it is diazotized and coupled with disodium 3-hydroxynaphthalene-2,7-disulfonic acid (R-salt).⁴⁴ For the reaction of aniline and various related compounds with 4-azobenzenediazonium fluoborate, see dimethylaniline, page 390. For the absorption after reaction with chloranil in chloroform, see Table 22.

Procedure—By 1-amino-8-naphthol-3,6-disulfonic acid. Acid copper-plating baths. To prepare the citrate solution, dissolve 300 grams of citric acid in 1 liter of water and adjust the pH to 6.5-7 with 10% potassium hydroxide solution.

Dilute a 10-ml. sample with 10 ml. of water. Add a 20 \times 20-mm. aluminum sheet and 1 ml. of 30% ammonium chloride solution. Let stand for 30 minutes and occasionally wipe the copper off the aluminum sheet. Add 10 ml. of 3% sulfuric acid and let stand for 5 minutes. Filter, and

³⁸ B. V. Ponomarenko, *Trudy Komissii Anal. Khim., Akad. Nauk S.S.S.R., Inst. Geokhim. i Anal. Khim.* 7, 289-94 (1956); V. Bakensky and J. Sule, *Korose Ochraua Materialu* 2, 65-8 (1958).

³⁹ I. M. Korenman and P. A. Ganiechev, *Uchenye Zapiski Gor'kov. Univ.* 24, 123-5 (1953).

⁴⁰ *ibid.*, 119-22.

⁴¹ K. W. Merz and A. Kammerer, *Arch. Pharm.* 286, 198-205 (1953).

⁴² V. Kratochvil, *Z. anal. Chem.* 183, 267-72 (1961).

⁴³ L. A. Zil'berg, *Gigiena Truda i Professional. Zabolevaniya* 4, 9, 47-8 (1960).

⁴⁴ J. L. Clipson and L. C. Thomas, *Analyst* 88, 971-2 (1963).

wash until the total volume of filtrate and washings is 100 ml. Add a piece of ice followed by 1 ml. of 10% sodium nitrite solution. After 5 minutes, add 2 ml. of 30% urea solution to destroy excess nitrous acid. Let stand for 45 minutes. Add 10 ml. of citrate solution. Add solid sodium bicarbonate until the rapid evolution of carbon dioxide ceases. Add 1 ml. of 1% 1-amino-8-naphthol-3,6-disulfonic acid solution containing a small amount of sodium bicarbonate. Add more solid bicarbonate until the evolution of carbon dioxide stops completely and there is an excess present. Add additional ice, if necessary. Remove excess carbon dioxide by bubbling air through the solution for 5 minutes. Add 1 ml. of 10% sodium bisulfite solution to prevent air oxidation and dilute to 250 ml. with a freshly prepared saturated sodium bicarbonate solution. Read against water at 536 $m\mu$.

Air absorbed in 1:110 hydrochloric acid. To a sample containing 0.001 mg. of aniline, add 5 ml. of 1:10 hydrochloric acid. Diazotize with 4 drops of 10% potassium nitrite solution and 1 drop of 5% potassium bromide solution. Let stand for 30 minutes with stirring. Neutralize with 3 ml. of 19.3% sodium carbonate solution and immediately add 2 drops of freshly prepared 1-amino-8-naphthol-3,6-disulfonic acid solution. Read at 536 $m\mu$ after 30 minutes.

By H-acid. To a 25-ml. sample containing 0.002-0.08 mg. of aniline per ml., add 1 ml. of 1:10 hydrochloric acid and 5 ml. of 2% sodium nitrite solution. Shake and place in ice for 30 minutes, shaking every 10 minutes. Add 6 ml. of 2% sodium bicarbonate solution and shake again. Add 0.2 ml. of 0.5% solution of H-acid and dilute with 50 ml. of water. Read at 600 $m\mu$ after 40 minutes.

By phenol. Aromatic amino acids. To a 25-ml. sample containing 0.002-0.1 mg. of aniline per ml., add 1 ml. of 1:10 hydrochloric acid, 5 ml. of 2% sodium nitrite solution, 6 ml. of 2% sodium bicarbonate solution, and 4 ml. of 1% phenol solution. Heat for 5 minutes at 50° and cool. Read at 470 $m\mu$ after 10 minutes.

By p-phenylenediamine. Sprout retardant. Adjust a 20-ml. sample to pH 5-6 with hydrochloric acid or acetic acid. Add 2 ml. of 0.2% p-phenylenediamine solution and 2 ml. of 2.5% potassium persulfate solution. After 10 minutes, add 3 ml. of 8% sodium hydroxide solution. Extract the solution for 2 minutes with 10 ml. of carbon tetrachloride. Read at 500 $m\mu$.

By R-salt. Air. As a reagent, dissolve 0.8 gram of R-salt in 100 ml. of boiling water and adjust to pH 7.5-8.5 with 10% sodium carbonate solution. Filter.

Pass a 6-liter sample at 1.5 liters per minute through 1:20 hydrochloric acid. Dilute the absorbate to 10 ml. with 1:20 hydrochloric acid. To 5 ml., add 0.5 ml. of 3.5% sodium nitrite solution. After 20 minutes, add 2 ml. of 10% sodium carbonate solution. Add 0.5 ml. of the reagent, mix, and add 2 ml. of 1:4 ammonium hydroxide. Compare with standards.

ACETANILIDE, ACETYLANILINE

Acetanilide is developed with nitrous acid and thymol.⁴⁵ The same reaction is applicable to many acetanilide derivatives.

Sample—Pharmaceutical preparations. Dissolve a sample containing 0.001-0.1 mg. of acetanilide in 20 ml. of 1:2 hydrochloric acid. Reflux for 5 minutes and neutralize with 15% sodium hydroxide solution or 15% potassium hydroxide solution. Dilute to 100 ml. To a 10-ml. aliquot, add 5 ml. of 0.5% sodium nitrite solution and 5 ml. of 1:110 hydrochloric acid. After 3 minutes, add 5 ml. of 1% thymol solution containing 1.5% of sodium hydroxide. Read after 2 minutes.

PARACETAMOL, 4'-HYDROXYACETANILIDE

Paracetamol is determined by reaction with 1-naphthol.⁴⁶ The sulfate ether and the glucuronate do not interfere. An alternative is reading in 0.04% sodium hydroxide solution at 257 m μ .⁴⁷

Procedure—Blood. To a 2-ml. sample, slowly add anhydrous granules of sodium sulfate until a friable mass is obtained. Prepare a 1.5% solution of pentyl alcohol in ethyl ether. Wash this with 40% sodium hydroxide solution, 1:9 hydrochloric acid, and water. Dry with anhydrous sodium sulfate before use. Extract the sample with 100-150 ml. of the solvent in a Soxhlet apparatus. Evaporate to about 60 ml. and extract with 5 ml. and 5 ml. of 0.4% aqueous sodium hydroxide. Combine the extracts and add 1.5 ml. of concentrated hydrochloric acid. Heat at 100° for 45 minutes, then cool to 25°.

⁴⁵ J. Richter, *Z. anal. Chem.* **142**, 277-9 (1954).

⁴⁶ J. R. Gwilt, A. Robertson and E. W. McChesney, *J. Pharm. Pharmacol.* **15**, 440-4 (1963).

⁴⁷ E. R. Brown and J. R. Gwilt, *Pharm. J.* **192**, 419-20 (1964).

As color reagent, add 10 mg. of potassium dichromate to 1 ml. of 5% solution of 1-naphthol in 95% ethanol. Add 1 ml. of 1:5 hydrochloric acid. After 3-5 minutes, dilute to 20 ml. with the 5% ethanolic naphthol solution.

Add 5 drops of reagent and 2.5 ml. of 40% sodium hydroxide solution to the cooled sample. After reaction for 2-3 minutes, saturate with solid potassium chloride. Extract with 5 ml. of butanol. Dry the extract with anhydrous sodium sulfate and read at 635 $m\mu$.

N-BUTYL ACETANILIDE

This is determined at the same time as butyl benzoate.⁴⁸ (Vol. IIIA, p. 381).

p-CHLOROACETANILIDE

Phenacetin samples are refluxed with 48% hydrobromic acid, the phenacetin converted to *p*-hydroxyaniline hydrobromide and the *p*-chloroacetanilide converted to *p*-chloroaniline hydrobromide. The mixture is made alkaline and the *p*-chloroaniline is extracted selectively with cyclohexane and read in the ultraviolet.⁴⁹ The procedure is sensitive to as little as 10 ppm. of *p*-chloroacetanilide in phenacetin. Caffeine and acetylsalicylic acid do not interfere. Starch interferes by the formation of dark-colored insoluble products, which must be removed. At 298 $m\mu$, 1 mg. of aniline shows as much absorbance as 0.69 mg. of *p*-chloroaniline.

Procedure—Phenacetin preparations. To a 2-gram sample, add 25 ml. of 48% hydrobromic acid. Add boiling chips to the flask, and reflux vigorously on a hot plate in a hood for 2 hours, using an air-cooled condenser. Remove the flask from the condenser and allow to stand in the hood until the fuming stops. Place in an ice bath and let stand for 5 minutes. Add 10 ml. of cyclohexane and 40 ml. of 25% sodium hydroxide solution. Let stand until cool. Transfer to a separatory funnel and rinse the flask thoroughly with 10 ml. of cyclohexane, adding the rinsing to the separatory funnel. Stopper the funnel and shake vigorously for 3 minutes. Allow the layers to separate and drain the aqueous layer into a second separatory funnel. Add 20 ml. of cyclohexane to the aqueous layer and shake as before. Discard the aqueous layer and drain the cyclohexane

⁴⁸ Morton Beroza, *Anal. Chem.* **25**, 112-15 (1953).

⁴⁹ W. B. Crummett, J. Simek and V. A. Stenger, *Anal. Chem.* **36**, 1834-7 (1964).

solution from the second funnel into the first. Allow the cyclohexane to drain into a clean 125-ml. separatory funnel. Wash the walls of the first funnel with 5 ml. of cyclohexane and add to the cyclohexane solution. Add 5 ml. of 25% sodium chloride solution to minimize emulsion formation and shake vigorously for 30 seconds. Discard the aqueous layer and repeat the extraction with the sodium chloride solution. Discard the aqueous layer.

Dilute the cyclohexane to 50 ml. Record the absorbance from 350 to 260 $m\mu$ on a recording spectrophotometer, using cyclohexane as a reference liquid. Draw a base line through the minimum at 268 $m\mu$ and tangent to the curve at 323 $m\mu$. Subtract the base line absorbance at 298 $m\mu$ from that indicated on the curve to obtain a net absorbance. Calculate as follows:

$$\begin{aligned} \text{net absorbance at 298 } m\mu \times \text{coefficient } C, 0.388 \text{ mg. per unit, } \times 1.33 \\ = \text{mg. of } p\text{-chloroacetanilide} \end{aligned}$$

RODILONE, *p,p'*-SULFONYLBISACETANILIDE

This is developed by the reaction with nitrous acid and thymol, shown for acetanilide, on page 381.

THIACETAZONE, TEBETHION, 4-FORMYLACETANILIDE THIOSEMICARBAZONE

Tebethion is developed in pharmaceutical preparations with nitrous acid and thymol.⁵⁰ Follow the procedure under acetanilide, page 381.

SALICYLANILIDE, *o*-HYDROXYBENZANILIDE

In varnish samples, the absorption of an alkaline extract of salicylanilide is measured at 336 $m\mu$.⁵¹ Although salicylanilide shows stronger absorption at 220 and 270 $m\mu$ than at 336 $m\mu$, these wave lengths are unsuitable because of interference of other varnish components.

As a method for *salicylanilide*, applicable to 20-100 mg., hydrolyze the sample with 50% alcoholic potassium hydroxide. Steam distil the aniline and diazotize. Neutralize excess nitrous acid with urea and couple with 5% thymol in 10% sodium hydroxide solution.⁵²

⁵⁰ J. Richter, *Z. anal. Chem.* **142**, 277-9 (1954).

⁵¹ M. H. Swann and M. L. Adams, *Anal. Chem.* **30**, 1807-8 (1958).

⁵² Maria Brzizka-Bak and Tadeusz Syrowatka, *Roczniki Państwowego Zakładu Hig.* **12**, 309-13 (1961).

Procedure—*Varnishes*. From a dropping bottle, weigh a sample of approximately 2.5 grams accurately into a separatory funnel containing 40 ml. of ethyl ether and mix. If the varnish precipitates, use benzene in place of ether. Add 20 ml. of a 2.5% sodium hydroxide solution, agitate thoroughly, and let stand to separate the layers. Draw off the lower aqueous layer into a second funnel containing 40 ml. of benzene and agitate. In cases where benzene is used in the initial funnel, use ether in the second. Repeat this extraction procedure with the sample, adding three additional 20-ml. portions of 2.5% sodium hydroxide solution. Draw off each aqueous layer without filtering from the second funnel and dilute the combined portions to 150 ml. with water. Heat at 80-85° for 1 hour, cool to room temperature, and dilute to 250 ml. with water. Mix thoroughly. Filter a 100-ml. aliquot and discard the first 50 ml. of filtrate. If the sample is completely free from cloudiness or separation, the filtration step may be eliminated.

Dilute a 5 ml. portion of the filtrate to 250 ml. with water and mix thoroughly. Read at 336 $m\mu$ against water. If the value of the absorbance is not between 0.2 and 0.5, use a new aliquot of the filtered sample of appropriate size. Apply cell corrections if necessary. Calculate as follows:

$$\% \text{ salicylanilide} = \frac{A_{336} \times 6250}{a_{336} \times b \times s.w. \times al.}$$

in which A_{336} is the absorbance after cell correction; a_{336} is the absorptivity obtained in calibration; b is the cell length in cm.; $s.w.$ is the sample weight; and $al.$ is the size of aliquot in ml.

ANTHRANILIC ACID, *o*-AMINOBENZOIC ACID

Anthranilic acid reacts to form colored products with *p*-dimethylaminobenzaldehyde⁵³ in 0.5 *N* acid. Indole gives a similar reaction with a different absorption. Tryptophan gives a color only at 11 *N* acid. For separation of *o*-aminobenzoic acid from related tryptophan metabolites, see kynurenine, page 494. This also applies to 3-hydroxyanthranilic acid.

Procedure—To 5 ml. of sample, add 1 ml. of 6% *p*-dimethylaminobenzaldehyde in 1:9 sulfuric acid. After 20 minutes, read at 420 $m\mu$. The same solution can be read for indole at 550 $m\mu$.

⁵³ D. Kupfer and D. E. Atkinson, *Anal. Biochem.* **8**, 82-94 (1964).

DIAPHENE

The differential absorbance of diaphene, a mixture of 5,4'-dibromosalicylanilide and 3,5,4'-tribromosalicylanilide, is determined by measuring a diaphene solution in methanol at pH 3 against a diaphene solution in methanol at pH 8.⁵⁴ Beer's law is followed for up to 5 mg. of diaphene per 100 ml. The absorbance of the solution at pH 8 decreases gradually on exposure to sunlight for 10 minutes. After that initial period, the absorbance does not change over a 3-hour period. The acid solution at pH 3 does not change absorbance upon exposure to sunlight. Ultraviolet light also affects the absorbance of the alkaline solution and not the acidic solution.

Procedure—Liquid soap. Prepare an acetic acid-methanol solution at pH 3 by dissolving 18 grams of glacial acetic acid in 90% methanol and diluting to 1 liter. Adjust to pH 3 with 1:110 hydrochloric acid. Adjust 90% methanol to pH 8 with 0.4% sodium hydroxide solution.

Dissolve a 5-ml. sample in 90% methanol and dilute to 100 ml. with 90% methanol. The final dilution should not contain more than 5 mg. of diaphene per 100 ml. To 5 ml. of this solution, add 20 ml. of 90% methanol and adjust the pH to 3 with 1:110 hydrochloric acid. Wash the electrodes with pH 3 acetic acid-methanol solution. Dilute to 50 ml. with the acetic acid-methanol solution and mix thoroughly.

To another 5-ml. portion of the sample solution, add 20 ml. of 90% methanol and adjust the pH to 8 with 0.4% sodium hydroxide solution or 1:110 hydrochloric acid. Wash the electrodes with 90% methanol at pH 8. Dilute to 50 ml. with 90% methanol at pH 8 and mix thoroughly.

Read the pH 3 solution against the pH 8 solution at 280 m μ :

$$\% \text{ Diaphene} = (A \times 50 \times 100)/(I \times V \times W \times 100)$$

where A is the differential absorbance measured; I is the differential absorbance index (10); V is the volume of sample solution in 50 ml. of the assayed solution; and W is the volume of the sample in ml. in the sample solution $\times 1000$ or weight in mg.

p-AMINO BENZOIC ACID

Diazotizing and coupling with *N*-1-naphthylenediamine gives a red complex suitable for reading.⁵⁵ Coagulation of the protein with hydro-

⁵⁴ S. A. Soliman and L. E. Harris, *J. Pharm. Sci.* **52**, 43-46 (1963).

⁵⁵ Richard T. Merwin, *J. Assoc. Offic. Agr. Chemists* **45**, 289-91 (1962).

chloric acid is satisfactory. For other possible methods of determining *p*-aminobenzoic acid, see comments in procaine, page 405, *et seq.*

Procedure—Feed. Slurry a 5-gram freshly ground sample 10 ml. of water and add 15 ml. of concentrated hydrochloric acid. Swirl occasionally while heating at 100° for 25 minutes. The solution darkens. Cool, dilute to 250 ml. with water, and let the particles settle. Dilute a 50-ml. aliquot to 100 ml. Add filter aid, and filter, discarding the first 10-15 ml. if turbid.

Take 10 ml. of sample and blank. Add 2 ml. of fresh 0.1% sodium nitrite solution to each. After 3 minutes, add 2 ml. of 0.5% ammonium sulfamate solution. After 2 minutes, add 1 ml. of water to the blank. To the sample, add 1 ml. of 0.1% *N*-1-naphthylenediamine hydrochloride solution, prepared fresh weekly and stored in a dark bottle. After 10 minutes, read at 545 μ and subtract the blank reading.

ANESTHESINE, BENZOCAINE, ETHYLAMINOBENZOATE

Anesthesine is diazotized and coupled with β -naphthol for determination.⁵⁶ Chlorohexadine dilactate does not interfere. An alternative is to react with vanillin in ethanol.⁵⁷

Procedure—By β -naphthol. Shake about 5 grams of ground tablets with 5 ml. of water. Add 40 ml. of ether and shake vigorously. Separate the ether layer after an hour and dilute to 50 ml. Evaporate a 10-ml. aliquot to dryness. Warm the residue with 10 ml. of 1:100 hydrochloric acid to dissolve. Cool, filter, and wash the dish and filter with 5 ml. and 5 ml. of water. Add 0.3 ml. of 10% sodium nitrite solution. After 2-3 minutes, add 1 ml. of 2% sodium hydroxide solution and mix. Add 5 ml. of 0.1% β -naphthol in 5% sodium hydroxide solution. After 30 minutes, dilute with water to 250 ml. Read at 460 μ against a blank. The color decreases 2% in an hour.

By vanillin. To 0.5-3 ml. of 1% ethanol solution, add 2 ml. of 1:2 hydrochloric acid, 4.5 ml. of 2.5% solution of vanillin in 95% ethanol, and dilute to 10 ml. for reading.

⁵⁶ Halina Siedlanowska, *Farm. Polska* **19** (10), 214-15 (1963).

⁵⁷ G. I. Luk'yanchikova, *Med. Prom. SSSR* **16** (9), 46-8 (1962).

p-AMINOSALICYLIC ACID

In phosphate buffer, the ultraviolet spectrum of *p*-aminosalicylic acid shows the maximum absorption at 265 and 299 $m\mu$ and the minimum absorption at 244 and 285 $m\mu$.⁵⁸ Beer's law is followed for 0.25-1 mg. %. Reaction with sodium nitrite in the presence of sodium carbonate produces a yellow color.⁵⁹ (Also see Vol. III, pp. 419-22, Vol. IIIA, pp. 395-6).

p-Aminosalicylic acid and a urinary metabolite, *p*-acetamidosalicylic acid, are converted by heating with dilute hydrochloric acid into *m*-aminophenol. The latter is determined by diazotized *p*-nitroaniline,⁶⁰ and is sensitive to 0.001 mg. per 10 ml. of the test substance and to 0.01 mg. of aminophenol.

Another metabolite, *p*-acetaminosalicyluric acid, is determined by *p*-aminobenzaldehyde. *p*-Aminosalicylic acid and *p*-acetaminosalicyluric acid are determinable by *N*-(2-diethylaminoethyl)-1-naphthylamine. The combination of these permits determination of all three.

Sodium *p*-aminosalicylate is determined by the indophenol reaction with dimethyl-*p*-phenylenediamine hydrochloride.⁶¹ The *p*-aminosalicylate is separable from *p*-aminophenol by paper chromatography. 4-Aminosalicylate is developed in serum or urine by *trans*-cinnamaldehyde whether or not isoniazid is present.⁶²

The complex of ferric ion and *p*-aminosalicylic acid at pH 2.5 is read at 500 $m\mu$.⁶³ *p*-Aminosalicylic acid is developed with vanillin in acid solution.⁶⁴ At pH 11, activated by 300 $m\mu$, the maximum fluorescence of *p*-aminosalicylic acid is at 405 $m\mu$.⁶⁵

Procedure—*Direct reading. Serum.* Dilute a 1-ml. sample with 0.9% sodium chloride solution to 6.25 ml. Precipitate a 1-ml. aliquot with 3

⁵⁸ R. Gosswald, *Naunyn-Schmiedeberg's Arch. exptl. Pathol. Pharmacol.* **222**, 220-2 (1954).

⁵⁹ Julia M. Ahon de la Vega, *Anales fac. farm. y bioquim., Univ. nacl. Mayor San Marcos* **1**, 33-41 (1950).

⁶⁰ Kiichiro Kakemi, Takaichi Arita, Hajime Yamashina and Ryoji Konishi, *J. Pharm. Soc. Japan* **82**, 540-2 (1962); cf. V. G. Smirnova, *Nauch. Raboty Khim. Lab. Gorkovsk. Nauch-Issledovatel. Inst. Gigieny Truda i Professional. Boloznei, Sbornik* No. 6, 64-8 (1957).

⁶¹ Konosuke Murai, *Yakuzaigaku* **20**, 210-13 (1960).

⁶² E. J. Hamilton, L. Eidus and Y. S. Lee, *Clin. Chim. Acta* **8**, 763-7 (1963).

⁶³ Rehati Charan Das and S. Aditya, *J. Indian Chem. Soc.* **37**, 557-61 (1960).

⁶⁴ Edward N. Deeb and Guy R. Citagliano, *J. Amer. Pharm. Assoc., Sci. Ed.* **44**, 182-5 (1955).

⁶⁵ Sidney Udenfriend et al., *J. Pharmacol. Exptl. Therap.* **120**, 26-32 (1957).

ml. of 96% ethanol. After 5 minutes, centrifuge at 3000 rpm. Read at 265 $m\mu$ against serum free from *p*-aminosalicylic acid.

Diazotized blood. Dilute a 1-ml. oxalated sample to 2 ml. with water. After hemolysis, add 2 ml. of 10% trichloroacetic acid solution and heat at 100° for 30 minutes. Plug with cotton and place in an ice bath. Add 3 ml. of water, 0.5 ml. of a stock standard solution containing 0.137 gram of the sodium salt of *p*-aminosalicylic acid, and 0.5 ml. of 10% sodium nitrite solution. After 3 minutes at 0°, add 2 ml. of 10% sodium carbonate solution. Let stand at room temperature for 15 minutes and dilute to 10 ml. with water. Read the yellow color with a blue filter and subtract the *p*-aminosalicylic acid added.

Urine. Dilute a sample 1:99 with water and develop a 2-ml. aliquot as described under "Blood."

Biological fluids. Dilute a sample to 2 ml. with water. Add 1 ml. of 10% trichloroacetic acid solution and follow the procedure under "Blood," starting at "... heat at 100° for 30 minutes."

By vanillin. Plasma. Dilute an appropriate sample, such as 2 ml. to 12 ml. with water. Add 4 ml. of 20% trichloroacetic acid. Shake vigorously, centrifuge, and filter the upper layer. To 8 ml. of the protein-free filtrate, add 1 ml. of 10% vanillin solution in 50% ethanol and 1 ml. of 1:6 sulfuric acid. Mix, let stand for 10 minutes, and read at 410 $m\mu$ against a reagent blank.

By trans-cinnamaldehyde. Serum. Isoniazid absent. To 2 ml. of serum, add 2 ml. of 25% trichloroacetic acid solution. After 15-20 minutes, centrifuge for 10 minutes and filter. To 2 ml. of filtrate, add 2 ml. of 4% *trans*-cinnamaldehyde solution in absolute ethanol. Read within the first 15 minutes at 390 $m\mu$.

Isoniazid present. Mix 2 ml. of serum with 0.2 ml. of 1:7 sulfuric acid. Shake, add 2.2 grams of sodium sulfate, and mix well. Extract with 20 ml. of 3 butanol:7 chloroform for 30 minutes. Filter through paper wet with the solvent mixture. Shake the filtrate with 3 ml. of 0.8% sodium hydroxide solution for 15 minutes. Centrifuge to separate the aqueous phase. Mix 1 ml. with 1 ml. of 1:20 hydrochloric acid. Add 2 ml. of 25% trichloroacetic acid solution and complete as with isoniazid absent.

Urine. Dilute 1:100, and treat as for serum with isoniazid absent or present.

IPC, *O*-ISOPROPYL-*N*-PHENYLCARBAMATE

This herbicide is determined by the aniline present after hydrolysis. Acid hydrolysis has been followed by extraction and determination by hypochlorite-phenol reagent.⁶⁶ Alkaline hydrolysis has been followed by determination with naphthylethylenediamine dihydrochloride.⁶⁷ In some cases, at least the plant tissue can be hydrolyzed directly, eliminating extraction, and amounts as low as 0.1 ppm. determined. The method is probably equally applicable to isopropyl *N*-(3-chlorophenyl)-carbamate.

Procedure—Place the sample containing 0.02-0.2 mg. of the herbicide in 100 ml. of 20% sodium hydroxide solution. Reflux for 4 hours. Connect the condenser by a glass U to a second vertical one. Turn off the water to the first condenser. Turn it on in the second one and distil the aniline into 20 ml. of 1:3 hydrochloric acid. The rate should be 10 ml. of distillate per minute for 5 minutes. Rinse down the condenser with 1:10 hydrochloric acid and dilute to 100 ml. with that concentration.

To 40 ml. of the diluted distillate, add 1 ml. of 2% sodium nitrite solution and let stand to diazotize for 20 minutes. Add 1 ml. of 10% sulfamic acid to destroy excess nitrite. After 15 minutes, add 5 ml. of fresh 2% solution of *N*-1-naphthylethylenediamine dihydrochloride and dilute to 50 ml. with 1:10 hydrochloric acid. Read at 560 $m\mu$ after 90 minutes.

DIMETHYLANILINE

Dimethylaniline forms a violet color by reaction with sodium chlorite in aqueous acid ethanol medium. The maximum color is obtained at pH 5.8-6.5 after 10 minutes.⁶⁸ A 10-fold excess of methylaniline and a 100-fold excess of aniline does not interfere. Beer's law is followed (cf. Vol. IV, pp. 199-200).

Dimethylaniline reacts with 4-azobenzenediazonium fluoborate to give

⁶⁶ Wilhelm E. Bissinger and Robert H. Fredenburg, *J. Assoc. Offic. Agr. Chemists* **34**, 812-16 (1951); cf. Leavitt N. Gard and Noland G. Rudd, *J. Agr. Food Chem.* **1**, 630-2 (1953).

⁶⁷ Marvin Montgomery and V. H. Freed, *ibid.* **7**, 617-18 (1959).

⁶⁸ G. Popa, E. Grigore and F. M. Albert, *Z. anal. Chem.* **193**, 324-6 (1963).

the blue color of *N,N*-dimethyl-*p*-(*p*-phenylazophenylazo)aniline.⁶⁹ This is read at 632 m μ . A similar reaction is given by aniline, 1- and 2-naphthylamines, α - and β -anthramines, and their *N*-alkyl and *N,N*-dialkyl derivatives. Differences in the rate of reaction permit differentiation between aniline derivatives and the more complex compounds. For the absorption after reaction with chloranil in chloroform see Table 22.

Procedure—Prepare a reagent solution containing 5% of sodium chlorite of 80% purity. To 0.2-2 ml. of ethanolic solution containing 0.03-0.3 mg. of dimethylaniline, add 3 ml. of the sodium chlorite reagent. Add 1-2 drops of 1:9 hydrochloric acid and dilute to 10 ml. Shake, and let stand for 10 minutes. Read at 533 m μ .

RHODANILINE

Rhodaniline, extracted from the air into acetic acid solution, forms a red color by oxidation with lead oxide.⁷⁰

Sample—*Air*. Wash a cotton plug with hot 10% acetic acid solution and dry. Draw the air through the dry plug at the rate of 10-15 liters per minute. Extract the cotton with 20 ml. of 50% acetic acid solution. To 1-5 ml. of the extract containing 0.0004-0.1 mg. of rhodaniline per ml., add 7 drops of a suspension of 200 mg. of lead oxide in 50 ml. of 25% acetic acid. Compare with standards.

o-AMINOPHENOL

o-Aminophenol (cf. Vol. IV, p. 201) is determined in 2-methyl-5-vinylpyridine by dissolving in *n*-heptane and extracting the *o*-aminophenol with 1:10 hydrochloric acid. The acid phase is buffered with ammonium acetate-acetic acid and the *o*-aminophenol is oxidized with hydrogen peroxide.⁷¹ The absorbance is read at 435 m μ . *p*-Tert-butylcatechol does not interfere. The method detects up to 0.005 mg. per ml.

Sample—*2-Methyl-5-vinylpyridine*. As a buffer, dissolve 200 grams of ammonium acetate in 500 ml. of water. Add 10 ml. of glacial acetic acid and dilute to 2 liters with water.

⁶⁹ Eugene Sawicki, James Noe and Francis T. Fox, *Talanta* **8**, 257-64 (1961).

⁷⁰ V. A. Vinogradova, *Gigiena Truda i Professional. Zabolevaniya* **4**, 56-7 (1960).

⁷¹ Kurt H. Nelson and M. D. Grimes, *Anal. Chem.* **30**, 1928-31 (1958).

Shake a 1-ml. sample containing up to 0.1% of *o*-aminophenol with 10 ml. of *n*-heptane and 8 ml. of 1:110 hydrochloric acid for 5 minutes and drain off the acid phase. Add 8 ml. of 1:110 hydrochloric acid to the heptane phase and shake again for 5 minutes. Combine the acid phases containing the extracted *o*-aminophenol. Add 30 ml. of buffer and 1 ml. of 30% hydrogen peroxide. Dilute to 50 ml. with the buffer. At 20 minutes after the addition of hydrogen peroxide, record the spectrum of the solution from 300 to 700 $m\mu$ against the buffer. Plot the wave length against the absorbance as shown in Figure 10. On the plotted spectrum, draw

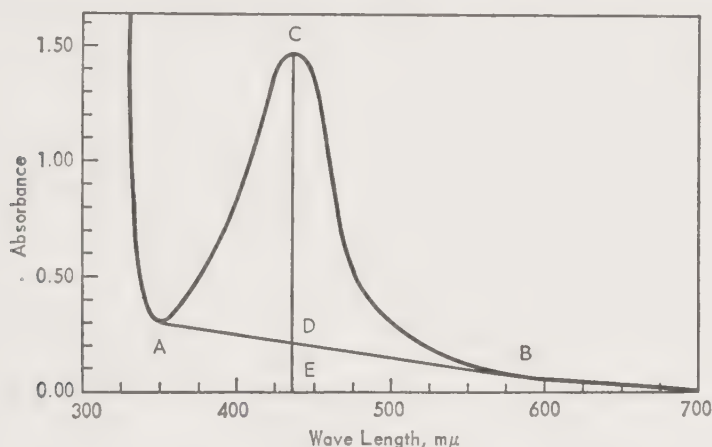


FIG. 10.

Spectrum of oxidized *o*-aminophenol, showing calculation of the absorbance at 435 M

the line AB from 350 to 585 $m\mu$. Draw a second line CDE perpendicular to the base at 435 $m\mu$. Measure the absorbance along CD. Read the mg. of *o*-aminophenol from the calibration curve and calibrate the weight per cent of *o*-aminophenol, using the following equation:

$$o\text{-aminophenol, weight \%} = A/M \times D \times 10$$

in which *A* is the weight of *o*-aminophenol; *M* is the volume of 2-methyl-5-vinylpyridine in ml.; and *D* is the density of 2-methyl-5-vinylpyridine in grams per ml., from Table 25.

m-AMINOPHENOL

In the determination of *m*-aminophenol in calcium aminosalicylate by the formation of a diazo compound, sulfuric acid is used in place of

TABLE 25. DENSITY OF 2-METHYL-5-VINYLPYRIDINE IN GRAMS PER MILLILITER

Temp., °C.	95% Pure	97% Pure	99% Pure	100% Pure
15.0	0.9606	0.9614	0.9622	0.9626
20.0	0.9563	0.9571	0.9579	0.9583
25.0	0.9520	0.9528	0.9536	0.9540
30.0	0.9478	0.9485	0.9493	0.9497

hydrochloric acid (cf. Vol. IV, p. 202). The absorbance values are lower with hydrochloric acid, and in the initial solution, it tends to produce a precipitate.⁷² The color is stabilized with sodium carbonate.

m-Aminophenol is isolated from sodium 4-aminosalicylate by paper chromatography and the aminophenol spot is developed with 4-dimethylaminobenzaldehyde.⁷³ Diazotized *p*-nitroaniline is also a reagent for *m*-aminophenol and *p*-aminosalicylate.⁷⁴ Beer's law is followed for 0.005-0.1 mg. of *m*-aminophenol. The color is stable for 1 hour.

In the presence of *p*-aminosalicylic acid, the sample is dissolved in methanol and precipitated with a large excess of ethyl ether.⁷⁵ The *m*-aminophenol remains in solution. It is then reacted with diazotized *p*-nitroaniline. *m*-Aminophenol is also developed by the complex formed with diethylene-*p*-phenylenediamine sulfate and potassium ferricyanide in ammonium hydroxide.⁷⁶

Both *m*-aminophenol and sodium aminosalicylate give a blue complex with phosphomolybdic acid. The intensity of the former is 90 times that of the latter, permitting differential estimation of *m*-aminophenol without isolation.⁷⁷ Unreacted *m*-aminophenol can be isolated from *p*-aminosalicylic acid and determined by ninhydrin.⁷⁸ For determination by titanium sulfate, see Volume IIIA, page 78. The maximum absorption is at 417 m μ .

Procedure—By diazotizing. Calcium aminosalicylate. A suitable sample is equivalent to 562 mg. of anhydrous calcium aminosalicylate or

⁷² R. B. Luers, Jr. and L. B. Stadler, *J. Pharm. Sci.* **51**, 910-11 (1962).

⁷³ T. Kowalczyk-Kaniewska, *Acta Polon. Pharm.* **19**, 341-4 (1962).

⁷⁴ Takanobu Itai and Hiroshi Igeta, *Bull. Natl. Hyg. Lab.* **72**, 81-5 (1951).

⁷⁵ M. Sanz, *Pharm. Acta Helv.* **27**, 270-1 (1952).

⁷⁶ O. Hrdý and H. Petříková, *Českoslov. farm.* **6**, 587-9 (1957).

⁷⁷ Tatjana Bičan-Fišter, *J. Pharm. Pharmacol.* **14**, 280-3 (1962).

⁷⁸ K. N. Gaiind, R. N. Dar and S. C. Bapna, *Indian J. Pharm.* **26**, 248-9 (1964).

500 mg. of aminosalicylic acid. To the sample, add 1.8 ml. of 4% sodium hydroxide solution and dilute to 80 ml. with water. Add 10 ml. of 1:10 sulfuric acid, dilute to 100 ml. with water, and mix. Within 150 seconds from the time the acid is added, transfer a 5-ml. aliquot of the solution to 50 ml. of water at 0.5° in an ice bath. Add 2.5 ml. of 1% sodium nitrite solution. Mix, and let stand in the ice bath for 3 minutes. Add 25 ml. of 10.5% sodium carbonate solution, mix, and warm at 25° for 15 minutes. Dilute to 100 ml. with water. Mix, and let stand at 25° for 3 hours. Filter, and read at 425-435 $m\mu$ against a water blank:

$$\text{per cent of } m\text{-Aminophenol} = A - (0.320/1.09)$$

in which *A* is the absorbance of the solution, and 0.320 is the absorbance correction factor representing color produced by other components present.

By 4-dimethylaminobenzaldehyde. Sodium 4-aminosalicylate. Prepare the 4-dimethylaminobenzaldehyde reagent by dissolving 0.1 gram in 10 ml. of ethanol and 0.1 ml. of 1:9 hydrochloric acid.

Dissolve a 2.5-gram sample in 8 ml. of methanol. Dilute to 50 ml. with ethyl ether and shake thoroughly. Apply 0.2-ml. aliquots to two strips, 50 cm. \times 2.5 cm., of Whatman No. 4 filter paper. Place the strips in a chromatography chamber saturated for 12 hours with an organic phase of butanol and 10% ammonium hydroxide solution. Develop for 12 to 24 hours by the ascending technic in the dark. Dry the chromatograms at room temperature and locate the 3-aminophenol spot with the 4-dimethylaminobenzaldehyde reagent. Cut out the spot and elute with 5 ml. of water for 5 minutes with shaking. Add 1 ml. of reagent and read at 420 $m\mu$ after 15 minutes.

As phosphomolybdate. Sodium 4-aminosalicylate. Dissolve a 50-mg. sample containing less than 2% of *m*-aminophenol in 10 ml. of 0.2 *M* acetate buffer for pH 6. Add 7 ml. of a 5% solution of phosphomolybdic acid. After 10 minutes, add 3 ml. of 1:13 aqua ammonia. Read at 720 $m\mu$ after 5 minutes against the blue color developed from an equal amount of pure sodium *p*-aminosalicylate.

By diazotized p-nitroaniline. Sodium 4-aminosalicylate. Mix 1 ml. of a 10% solution of the sample with 8 ml. of chloroform and 12 ml. of ether. Shake well and add 5 grams of anhydrous potassium carbonate. Shake for 3 minutes and filter after 10 minutes. To 2 ml. of the filtrate, add 2 ml. of water. Evaporate the organic solvents at 100°. Dilute the

residue to 10 ml., and add 3 drops of 0.3% *p*-nitroaniline hydrochloride solution and 1 drop of 0.7% sodium nitrite solution. Mix well and add 1 ml. of 13.6% sodium acetate trihydrate solution. After 1 minute, add 0.5 ml. of 4% sodium hydroxide solution and read at 500 $m\mu$.

By diethylene-p-phenylenediamine sulfate. p-Aminosalicylic acid. Dilute a 5-ml. sample containing 25-75 mg. of *m*-aminophenol to 10 ml. with 95% ethanol. Dilute to 250 ml. with water. To a 1-4 ml. aliquot in a separatory funnel, add 5 ml. of water, 2 ml. of a 0.2% solution of diethylene-*p*-phenylenediamine sulfate, 2 ml. of 1:20 ammonium hydroxide, 10 ml. of benzene, and 2 ml. of 2% potassium ferricyanide solution. Shake for 20 seconds. Drain the water layer. To the benzene layer, add 3 ml. of water and 2 ml. of 1:20 ammonium hydroxide. Shake for 20 seconds. Dry the benzene layer with sodium sulfate and read at 550 $m\mu$.

By ninhydrin. 4-Aminosalicylic acid. Dissolve about 0.5 gram of 4-aminosalicylic acid in 20 ml. of water and adjust the pH to 9. Extract the peroxide-free ether for 8 hours. Set aside the aqueous phase for determination of 4-aminosalicylic acid (p. 387). Evaporate the ether extract to dryness and take up the *m*-aminophenol in 5 ml. of water. Add 5 ml. of 1:14 ammonium hydroxide, 0.2 ml. of 2% calcium chloride solution, and 5 ml. of a fresh 0.05% solution of ninhydrin. Heat at 70° for 5 minutes with shaking and cool. Dilute to 25 ml. and read at 505 $m\mu$ against a reagent blank.

p-AMINOPHENOL

Oxidation of *p*-aminophenol by cerium perchlorate in perchloric acid yields a purple compound that is probably a phenoxazine derivative⁷⁹ (cf. Vol. IV, p. 203). The reaction is specific for *p*-aminophenol. It also gives this color with ferric ion or silver ion as reagent.

Although diphenylamine forms a purple color when treated with ceric perchlorate, the color turns brown within seconds and fades. 4-Amino-1-naphthol forms a light brown color and does not interfere. Autooxidized *p*-aminophenol does not react with the ceric perchlorate to produce a purple color. By this method, *p*-aminophenol can be determined in the presence of the *m*- and *o*-isomers. Beer's law is followed for 0.5-5 mg. of *p*-aminophenol at 10 and 15 minutes, but not at 20 and 30 minutes.

p-Aminophenol forms a blue color when treated with 8-quinolinol.

⁷⁹ George G. Guilbault, *Anal. Chem.* **35**, 828-30 (1963).

This color fades after 30 minutes. *o*-Aminophenol and *m*-aminophenol do not react with the reagent. Beer's law is followed.⁸⁰ *p*-Aminophenol may be determined in the presence of the *m*- and *o*-isomers, with ninhydrin in potassium cyanide solution.⁸¹ Substituted *p*-phenylenediamines and *p*-aminophenols, such as *p*-toluenediamine sulfate, *o*-nitro-*p*-aminophenol and *p*-aminodiphenylamine, also give a blue color with ninhydrin. Nitro-*p*-phenylamine, *o*-aminophenol, *m*-aminophenol, *o*-phenylenediamine, *m*-phenylenediamine, aniline, *p*-aminobenzoic acid, *p*-bromoaniline, *p*-nitroaniline, and 4,6-dinitro-2-aminophenol do not react with ninhydrin. The color is stable for 1 hour. This method is also applicable to *p*-phenylenediamine in the presence of its meta and ortho isomers.

p-Aminophenol, 2,5-diaminotoluene, and *p*-phenylenediamine are determined simultaneously by conversion into diacetyl derivatives.⁸² Beer's law is followed to within $\pm 1\%$ in the range of 5-15 mg. per liter. For determination by titanium sulfate, see Volume IIIA, page 78. The maximum absorption is at 418 m μ .

Procedure—*As diacetyl derivatives. Hair dyes and rinses. p*-Aminophenol, *p*-phenylenediamine and 2,5-diaminotoluene, simultaneously. For the preparation of the diacetyl derivatives of the sample, follow the procedure under 2,5-diaminotoluene in Volume IV, page 229, starting with "Dissolve the sample in 6 ml. of 1:5 hydrochloric acid . . ." and ending with "Dry the residue at 100° to a constant weight." Develop as diacetyl derivatives.

Dissolve the weighed, mixed diacetyl derivatives of the sample in a known volume of 95% ethanol. Evaporate the alcohol from an aliquot in an acetylation flask at 100° with the aid of an air jet. This should contain approximately 25 mg. of derivatives. Add 10 ml. of water and a few carborundum chips to the residue. Place the flask in a transite board with a 1.5 inch diameter circular opening. Connect to a water-cooled condenser and heat with a small gas flame until solution is complete. Disconnect the condenser and connect a 10-ml. Dean and Stark distilling trap to the flask. Completely fill the trap with toluene, adding 5 ml. in excess, and allow it to overflow into the flask. Connect the trap to a water-cooled condenser and heat until all the water is distilled. Disconnect the flask, stopper, and let stand for 2 hours at room temperature. Filter through paper moistened with toluene. Rinse the flask and filter with two

⁸⁰ P. A. Caccia, *ibid.* **31**, 1306-8 (1959).

⁸¹ R. Sums, A. Levy and D. E. Dean, *ibid.* **36**, 636-7 (1964).

⁸² S. H. Newburger, *J. Assoc. Offic. Agr. Chemists* **37**, 793-6 (1954).

5-ml. portions of toluene. Evaporate the filtrate at 100° under an air jet and reserve the residue containing diacetyl-*p*-aminophenol. Return the filter paper to the acetylation flask and heat under a jet of air until all the toluene is evaporated. The residue is diacetyldiamines. Dissolve each residue in hot 95% ethanol and filter. Cool to room temperature, dilute to 100 ml. with ethanol and mix the two solutions. Dilute an aliquot to a concentration of 10 mg. per liter of ethanol. Read at 245, 254, and 265 $m\mu$. Calculate the composition of the sample from the absorbances of the sample and standards at the following wave lengths:

diacetyl- <i>p</i> -aminophenol	245 $m\mu$
diacetyl-2,5-diaminotoluene	254 $m\mu$
diacetyl- <i>p</i> -phenylenediamine	265 $m\mu$

Calculate the diacetyl compound as free amine bases as follows:

- 1 mg. of diacetyl-*p*-aminophenol = 0.565 mg. of *p*-aminophenol
 1 mg. of diacetyl-*p*-phenylenediamine = 0.563 mg. of *p*-phenylenediamine
 1 mg. of diacetyl-2,5-diaminotoluene = 0.592 mg. of 2,5-diaminotoluene

By ninhydrin. As a buffer, dissolve 21 grams of citric acid in water, add 200 ml. of 0.4% sodium hydroxide solution and dilute to 1 liter with water. To prepare the potassium cyanide reagent, dilute 5 ml. of 0.065% potassium cyanide solution to 250 ml. with methyl Cellosolve. Prepare the ninhydrin reagent by mixing 250 ml. of the potassium cyanide reagent with 50 ml. of 5% ninhydrin solution in methyl Cellosolve.

Dilute a sample containing up to 4 mg. of *p*-aminophenol to 1 ml. with water. Add 0.5 ml. of the citrate buffer and 1.2 ml. of the ninhydrin reagent. Heat for 15 minutes at 100° . Cool and dilute to 100 ml. Read immediately at 555 $m\mu$.

By 8-quinolinol. To prepare the reagent, dissolve 100 mg. of 8-quinolinol in 1 ml. of glacial acetic acid and dilute to 10 ml. with water. Dilute a sample containing 0.001-0.01 mg. of *p*-aminophenol to 1 ml. and add 1 ml. of the 8-quinolinol reagent. Let stand at room temperature for 5 minutes. Add 2 ml. of 0.4% sodium hydroxide solution and mix. Read at 620 $m\mu$ against a reagent blank after 20 minutes.

By ceric perchlorate. As a reagent, dissolve 4 grams of ceric hydroxide in 250 ml. of 72% perchloric acid and dilute to 1 liter with water. Store in a dark bottle and standardize from time to time against arsenious oxide. Add a sample containing 5-50 mg. of *p*-aminophenol to 10 ml. of

reagent. Stir, and let the purple color develop for 15 minutes. Read at 560 m μ .

ACETYL-*p*-AMINOPHENOL

After separation from *p*-aminophenol on an ion-exchange column, the acetaminophenol is read in the ultraviolet.⁸³ Caffeine does not interfere. An alternative is to separate, diazotize, and develop with *N*-(1-naphthyl)-*N'*-diethylpropylenediamine.⁸⁴

Sample—Tablets. First extract acetylsalicylic acid and acetyl-*p*-aminophenol with ether. Then extract the acetylsalicylic acid from the ether extract with sodium bicarbonate solution. Evaporate the ether extract, take up in water, and determine by *N*-(1-naphthyl)-*N'*-diethylpropylenediamine.

Alternatively, extract the acetyl-*p*-aminophenol from the sample with methanol and evaporate the extract. Develop with the above diamine.

Procedure—In the ultraviolet. Take a 10-ml. aliquot representing approximately 10 mg. of acetylaminophenol. Pass through a short column of Amberlite IR-120 in the acid form to remove the *p*-aminophenol. When the solution and washings reach the top of the column, elute with 100 ml. of water. This eluate contains *N*-acetyl-*p*-aminophenol, flavors, colors, etc. Read at 244 m μ . The *p*-aminophenol can be eluted from the column with alkali for determination by methods which precede this one.

By 1-naphthol. Hydrolyze 10 ml. of the eluate with 2 ml. of concentrated hydrochloric acid. Develop with two drops of fresh 10% solution of 1-naphthol in ethanol and 30% sodium hydroxide. Read at 590 m μ after 3-5 minutes. Results agree well with direct reading in the ultraviolet.

By *N*-(1-naphthyl)-*N'*-diethylpropylenediamine. Reflux the sample in 15% trichloroacetic acid and dilute to 50 ml. with the same. Add 3 ml. to 2 ml. of 15% trichloroacetic acid and 1 ml. of 0.5% sodium nitrite. Shake, and after 1.5 minutes, add 1 ml. of 0.5% ammonium sulfamate. Shake, and add 2 ml. of a solution of 0.05 gram of *N*-(1-naphthyl)-*N'*-

⁸³ K. Thomas Koshy and John L. Lach, *Drug. Std.* **28**, 85-7 (1960); *J. Pharm. Sci.* **50**, 113-18 (1961).

⁸⁴ Marcel Mouton and Monique Masson, *Ann. Pharm. Franc.* **18**, 759-62 (1960).

diethylpropylenediamine in 50 ml. of water and 10 ml. of amyl alcohol. After 30 minutes, withdraw 1 ml. of the amyl alcohol layer and dilute with 9 ml. of amyl alcohol. After 10 minutes, read at 590 m μ .

2,4-DIAMINOPHENOL

Potassium persulfate oxidizes 2,4-diaminophenol to a red 2-amino-4-quinoneimine, which is read at 500 m μ .⁸⁵ The oxidation is carried out at pH 4.5. Beer's law is followed over range 0.01-0.25 mg. per ml. In the presence of similar amounts of *m*-nitroaniline or *m*-phenylenediamine, there are errors of 5 and 10%, respectively. The method is sensitive to 0.01 mg. of diaminophenol.

Procedure—To a 2-ml. sample in 1:24 hydrochloric acid containing 0.01-0.25 mg. of 2,4-diaminophenol dihydrochloride per ml., add 1 ml. of 0.2% potassium persulfate solution and 2 ml. of 8.2% sodium acetate solution. After 10 minutes, dilute to 10 ml. with water and read at 500 m μ against water.

4-AMINODIPHENYL

A procedure for 4-aminodiphenyl involves diazotization and coupling of an acid extract with R-salt, disodium 2-naphthol-3,6-disulfonate. Chromatography on filter paper discs separates the 4-aminodiphenyl derivative. The 4-amino-diphenyl-R-salt band is eluted in a minimum of sodium hydroxide for reading.⁸⁶

Procedure—*Technical diphenylamine or residues from the distillation of diphenylamine.* Boil a 1-gram finely-ground sample with 5 ml. of 1:24 hydrochloric acid for 1 minute and cool the suspension in ice water. Add 0.2 ml. of 3.5% sodium nitrite solution with mixing. After 2 minutes, pour the suspension into a mixture of 2 ml. of 1.7% R-salt solution and 5 ml. of 10.5% sodium carbonate solution. Let stand for 5 minutes. Add 5 ml. of 4% sodium hydroxide solution and mix thoroughly.

Place six 32-cm. Whatman No. 1 filter papers on a 6 \times 6-inch glass plate. Cover with another plate of similar size having a $\frac{3}{16}$ - $\frac{1}{4}$ -inch hole in its center. Introduce the sample solution to the pad of filter papers through

⁸⁵ D. V. Parke, *Analyst* **86**, 12-15 (1961).

⁸⁶ H. E. Stagg and R. H. Reed, *Analyst* **82**, 503-6 (1957).

the hole. Follow the sample with 4% sodium hydroxide solution as the developing solvent.⁸⁷

After the chromatogram is fully developed and while the pad of filter papers is still wet, cut off the outer zones to leave the inner zone on a disc of paper approximately 17 cm. in diameter. Place this disc on the center of a square sheet of methyl methacrylate measuring 25 cm. \times 25 cm. \times 1 cm., in which a circular channel 0.5 cm. deep, 1 cm. wide, and having an internal diameter of 18.5 cm. was cut. In the floor of the channel, drill a hole 0.5 cm. in diameter. Place a glass plate 25 cm. \times 25 cm. with a central hole of 1 cm. diameter symmetrically on top of the paper disc and weigh down with two steel blocks weighing 5 lbs. each. Support the plates on a flat wooden block so that a beaker may be placed beneath the hole in the lower plate. Fit a calcium chloride tube to the hole in the upper glass plate by means of a rubber stopper and fill the tube with 0.4% sodium hydroxide solution to elute the 4-aminodiphenyl zone from the paper disc. As the eluting solvent soaks into the paper, the eluted dye is collected in the beaker and is complete when approximately 70 ml. are collected. Dilute the eluate to 100 ml. with 0.4% sodium hydroxide solution and read against a water blank.

PHENACETIN, *p*-ETHOXYACETANILIDE, *p*-ACETOPHENETIDIN

Acid hydrolysis of *p*-acetophenetidide produces *p*-phenetidine, which is diazotized and coupled with β -naphthol in alkaline solution to give an orange-red color read at 470 $m\mu$.⁸⁸ (cf. Vol. IV, pp. 204-5). Antihistamines, caffeine and salicylates do not interfere, and therefore, caffeine and phenacetin may be determined in a mixture of these components without separation or extraction. Alternative coupling agents are thymol⁸⁹ or phloroglucinol.⁹⁰ The hydrolysis product may also be condensed with *p*-dimethylaminobenzaldehyde for reading at 412 $m\mu$.⁹¹

Use of hydrochloric acid sometimes results in incomplete hydrolysis. Dilute sulfuric acid is therefore often used. Acetone dissolves the red-colored *p*-ethoxybenzediazonium 2-naphthol and also keeps any unreacted β -naphthol in solution to prevent turbidity. Certain diazotizable

⁸⁷ W. G. Brown, *Nature* **143**, 377-8 (1939).

⁸⁸ F. J. Bandelin and R. E. Pankratz, *Anal. Chem.* **28**, 218-21 (1956); R. E. Pankratz and F. J. Bandelin, *J. Am. Pharm. Assoc.* **45**, 364-6 (1956).

⁸⁹ C. M. P. Wirth, *Pharm. Acta Helv.* **35**, 575-7 (1960).

⁹⁰ Giulio Di Bacco, *Boll. chim. farm.* **93**, 366-9 (1954).

⁹¹ J. G. Devi and M. L. Khorana, *Indian J. Pharm.* **15**, 168-71 (1953).

primary aromatic amines such as sulfonamides, *p*-aminophenol, and acetanilide interfere but may be separated by preliminary extraction. An alternative coupling solvent is acetone, resulting in an absorption maximum at 546 m μ .⁹²

The color reactions of phenacetin are as follows: manganese dioxide 570-580 m μ , potassium 1,2-naphthoquinone-4-sulfonate 490-525 m μ and calcium hypochlorite and phenol 630 m μ .⁹³ In the usual concentrations in analgesic tablets, aminopyrine, phenazone, caffeine, monochlorosalicylacetate, barbital, and codeine do not interfere.

Phenacetin is quantitatively converted to nitrophenetidin by nitration and saponification with sodium hydroxide. The nitrophenetidin is soluble in alkaline solution and is read at 445 m μ .⁹⁴ Heat is necessary for the rapid and complete hydrolysis of nitrophenacetin to nitrophenetidin. Beer's law is followed for 1-5 mg. of phenacetin. Caffeine does not have to be separated. The usual tablet contains 5 times as much phenacetin as caffeine. Analysis of tablets by this method produces a concentration of 5 mg. of phenacetin per 100 ml. of final solution and 1 mg. of caffeine per 100 ml. of final solution.

In mixtures of acetylsalicylic acid, caffeine, and acetophenetidin, the acetophenetidin is eluted from a Celite column with isopropyl ether, and caffeine is eluted with chloroform. The acetophenetidin is then read at 290 m μ in the isopropyl ether eluate.⁹⁵

When a solution in ethanol is oxidized by adding aqueous chloramine, the color is yellow.⁹⁶ Pyramidon gives the same color. Aspirin, caffeine, methylcaffeine, and codeine do not interfere. Phenacetin and pyramidon are differentiated because the color of the latter is not destroyed by adding hydrochloric acid. An alternative oxidizing agent is acid dichromate.⁹⁷ Then aminopyrine and meprobamate interfere. Phenacetin is read in isopropyl ether at 290 m μ or in chloroform at 250 m μ .⁹⁸

Tablets containing salicylamide, acetylsalicylic acid, caffeine, and phenacetin are disintegrated in 2% sodium hydroxide solution. After

⁹² J. G. Devi and M. L. Khorana, *ibid.* **15**, 193-5 (1953).

⁹³ A. Ulebelen, *Rev. fac. sci. univ. Istanbul Ser C* **24**, 145-61 (1959).

⁹⁴ L. I. Rappaport, *Aptechnoe Delo* **6**, No. 3, 21-8 (1957); Lee Kum-Tatt and Chan Chian-Seng, *J. Pharm. and Pharmacol.* **12**, 624-30 (1960).

⁹⁵ Gordon Smith, *J. Assoc. Offic. Agr. Chemists* **39**, 624-9 (1956).

⁹⁶ B. N. Afanas'ey and A. I. Kruzhevnikova, *Zhur. Anal. Khim.* **12**, 143-5 (1957).

⁹⁷ R. García Villanova, J. Thomas, F. Bosch and J. M. Sune, *Ars. Pharm.* **3**, 127-37 (1962).

⁹⁸ Arias C. Josefina, *Anal. fac. farm. y bioquim., Univ. uel. mayor San Marcos (Lima)* **6**, 519-23 (1955).

filtration and appropriate dilution, salicylamide is read at 329 $m\mu$, acetylsalicylic acid at 300 $m\mu$, and caffeine at 273 $m\mu$, with corrections for the prior two ingredients. Then phenacetin is read at 250 $m\mu$ in ethanol, with corrections for the other three ingredients.⁹⁹

Procedure—*Mixtures of acetylsalicylic acid, acetophenetidine, and caffeine. By diazotization and coupling with β -naphthol.* As a buffer, dissolve 5 grams of sodium bicarbonate and 1 gram of sodium hydroxide in 100 ml. of water. Weigh a sample containing approximately 0.1 gram of phenacetin. Add 20 ml. of 1:9 sulfuric acid and heat at 100° for 3 hours. Cool to room temperature and dilute to 100 ml. with water. Dilute a 10-ml. aliquot to 200 ml. with water. To a 10-ml. aliquot of this dilution, add 15 ml. of water, 1 ml. of 1:10 hydrochloric acid, and 1 ml. of 1% sodium nitrite solution. After 3 minutes, add 1 ml. of 5% ammonium sulfamate solution and let stand for 1 minute. Add 20 ml. of the sodium hydroxide-sodium bicarbonate buffer and 1 ml. of 1% β -naphthol solution in acetone. Mix well and let stand for 15 minutes. Add 40 ml. of acetone, mix, and dilute to 100 ml. with water. Read at 470 $m\mu$.

By diazotization and coupling with thymol. Aspirin and caffeine present. Powder a sample of tablets and take a portion containing about 0.1 gram of phenacetin. Shake with 50 ml. of 95% ethanol and filter through paper. Discard the first 15 ml. To a 0.5-ml. aliquot of the filtrate, add 5 ml. of concentrated hydrochloric acid and evaporate to about 1 ml. Dilute with water to 5 ml. and chill in an ice bath. Add 1 ml. of 0.5% sodium nitrite solution. After 10 minutes, add 1 ml. of 1:45 sulfuric acid. Add 6.5 ml. of a 0.01% solution of thymol in 95% ethanol and 6.5 ml. of 4% sodium hydroxide solution. Shake, dilute to 50 ml. with water, and read at 500 $m\mu$.

As nitrophenetidin. Caffeine present. Dissolve the sample in chloroform and evaporate an aliquot containing 1-5 mg. of phenacetin at 100°. Add 2 ml. of 1:1 nitric acid to the residue and let stand for 30 minutes at room temperature. Add 10 ml. of 10% sodium hydroxide solution and heat at 100° for 30 minutes. Dilute to 100 ml. with water and read the nitrophenetidin at 445 $m\mu$ against water.

Caffeine and aspirin present. Dissolve a sample, which typically may contain 0.23 gram of aspirin, 0.16 gram of phenacetin, and 0.03 gram of

⁹⁹ G. Machek, *Sci. Pharm.* **29**, 73-80 (1961).

caffeine, in 500 ml. of chloroform. Extract a 10-ml. portion with 15 ml. of 6% sodium bicarbonate solution. Extract the sodium bicarbonate solution with three 10-ml. portions of chloroform and wash the combined chloroform extracts with 3 ml. of 6% sodium bicarbonate solution. Evaporate the extract to dryness. Add 2 ml. of 1:1 nitric acid to the residue and let stand at room temperature for 30 minutes. Follow the procedure for phenacetin in the presence of caffeine, starting at "Add 10 ml. of 10% sodium hydroxide . . ."

In the ultraviolet. Acetylsalicylic acid, caffeine, and acetophenetidin present. Tablets. The absorbance of the isopropyl ether in a 1-cm. silica cell at 276 $m\mu$ against water should not be more than 0.100. If the absorbance exceeds that value, purify the ether as follows: To 2 liters of isopropyl ether, add 150 grams of sodium hydroxide pellets and shake vigorously at least once per hour during the day. Let stand overnight. Distil the liquid in all-glass apparatus, using an asbestos-covered electric hot plate or heating mantle and a glass-stoppered receiving bottle. When the depth of ether drops to approximately 1 cm. in the distilling flask, stop heating and remove the flask promptly, as there is risk of explosion if the material is heated near dryness.

The absorbance of the chloroform in a 1-cm. silica cell at 276 $m\mu$ against water should not exceed 0.0500. To prepare the sodium bicarbonate solution, add 3 grams of sodium bicarbonate to 45 ml. of water previously cooled to 15° or lower. Stir to dissolve and add 2-3 drops of 1:3 hydrochloric acid.

Weigh a powdered sample containing approximately 20 mg. of caffeine. Add 8 ml. of water cooled to 15° or lower, and mix. Add 12 ml. of cold sodium bicarbonate solution and mix carefully. Extract immediately with five 25-ml. portions of chloroform. Wash each portion through a separatory funnel containing 3 ml. of cold sodium bicarbonate solution and filter through loosely packed cotton in the stem. Concentrate the combined extract to 10 ml. in a gentle air current without boiling. If the solution is evaporated to less than 10 ml., dilute to 10 ml. with chloroform. Take up in isopropyl ether. Dilute the combined solution and washings to 100 ml. with isopropyl ether.

Prepare the chromatographic tube by fusing a 5.5-6-cm. length of 6-8 mm. glass tubing to the rounded end of a 20-cm. length of 25 mm. tubing. Constrict the stem slightly 1.75-2 cm. below the joint. Cut fine glass wool into short lengths and pack tightly into the stem of the tube above the constriction. Support the tube vertically and attach a rubber

tube with a pinch clamp to the outlet to regulate the flow during packing. Mix 10 grams of Celite No. 545 with 45 ml. of isopropyl ether. Add 10 ml. of water and mix until the solid appears uniform. Transfer to the tube in approximately 5 portions and pack down each portion with a glass plunger. The rate of flow through the finished column should be 8-18 drops per 10 seconds when the free liquid at the top is approximately 5 cm. high. After packing, remove the rubber tube and wash the stem with isopropyl ether to remove any rubber residue. When the free liquid disappears at the top of the top, discard all liquid collected and place a 100-ml. volumetric flask at the bottom of the tube so that the tip of the tube extends into the flask. Pipet a 5-ml. aliquot of the sample onto the top of the column. When the liquid disappears, add 10 ml. of isopropyl ether, washing down the inside of the tube. When the liquid disappears, elute with 60-65 ml. of isopropyl ether, maintaining a liquid column not more than 7 cm. high. When the last of the isopropyl ether disappears, wash the tip of the tube with isopropyl ether and replace the volumetric flask with another one.

Add 70-75 ml. of chloroform to the column, maintaining a liquid height of not more than 7 cm. When the elution is complete, wash the tip of the tube with chloroform.

To determine phenacetin, dilute the isopropyl ether eluate to 100 ml. with isopropyl ether and read at 290 $m\mu$ against isopropyl ether at 4 minutes after placing the solution in the cell. To determine caffeine, dilute the chloroform eluate to 100 ml. with chloroform and read at 276 $m\mu$ against the chloroform-isopropyl ether blank at 4 minutes after the chloroform is placed in the cell. To prepare the chloroform-isopropyl ether blank, dilute 12 ml. of isopropyl ether to 50 ml. with chloroform.

Dichromate oxidation. Dissolve the sample in 200 ml. of 1:3 hydrochloric acid. Boil for 2 minutes to hydrolyze. Dilute an aliquot of 3-6 ml. containing 0.75-1.5 mg. to 6 ml. with 1:3 hydrochloric acid. Add 0.1 ml. of 0.4% potassium dichromate solution. Read after 150 minutes.

ETHYL AMINOBENZOATE, BENZOCAINE, ANESTHESINE

Primary, secondary, and tertiary amines give characteristic color reactions with acetic acid and lead dioxide. Benzocaine gives a red complex that dissolves in ether to give an orange-red color.¹⁰⁰

¹⁰⁰ J. Kolsek, *Z. anal. Chem.* **140**, 426-9 (1953).

Procedure—Dissolve the sample in 10% acetic acid solution and take an aliquot containing 0.25-1 mg. of anesthesine. Dilute to 5 ml. with water and add a small amount of lead oxide. Shake for 3 minutes to develop the red color. Add 120 ml. of ether, shake, and filter. Read 10 minutes after the addition of the oxide.

PROCAINE, NOVOCAINE, ETHOCAINE, DIETHYLAMINOETHYL
ESTER OF *p*-AMINOBENZOIC ACID

Yellow sodium 1,2-naphthoquinone-4-sulfonate reacts with a primary amine in alkaline solution to yield a highly colored red or orange-red product, an imine. The solution is made acidic with buffer and the excess yellow reagent is bleached with thiosulfate.¹⁰¹ Butethamine, propoxycaine, butacaine, and naepaine all produce color with the maximum at the same wave length of 483 $m\mu$ and the same intensity as procaine. Metabutethamine and metabutoxycaine also produce color with different absorption characteristics because of the primary amine in a position meta to the ester linkage. Those in the first group have the amine in a position para to the carboxylic ester linkage.

Absorption curves for meta amino compounds are less intense, and the maxima occur at a lower wave length of 478 $m\mu$. Meprylcaine, tetracaine, piperocaine and lidocaine, all with secondary and tertiary amine group, do not form color. The procedure is applicable to 0.02-1 mg. per 5 ml. of solution. There is a slight deviation from Beer's law.¹⁰²

In alkaline solution, procaine, cocaine, and tetracaine, are determined simultaneously in the ultraviolet.¹⁰³ In 0.12% sodium hydroxide solution, there is a single maxima at 226 $m\mu$, 264 $m\mu$, and 280 $m\mu$ for cocaine, procaine, and tetracaine, respectively. Both procaine and tetracaine absorb slightly at 226 $m\mu$. The absorption of cocaine at 264 $m\mu$ and 280 $m\mu$ is negligible. Although procaine and tetracaine absorb appreciably at each other's maximum, neither interferes seriously. Beer's law is followed for up to 15 ppm. for tetracaine and procaine and up to 30 ppm. for cocaine, at the wave lengths above. Egonine, milk sugar, starch, mannitol, and magnesium sulfate do not interfere.

¹⁰¹ Edward G. Feldmann, *J. Am. Pharm. Assoc.* **48**, 197-201 (1959).

¹⁰² M. Z. Barakat, S. K. Shehab and M. El-Shabrawi, *Drug Standards* **27**, 27-30 (1959).

¹⁰³ M. J. Pro, R. A. Nelson, W. P. Butler and A. P. Mathers, *J. Assoc. Off. Ag. Chemists* **39**, 857-64 (1956).

When added to diazotized procaine penicillin, *N*-(1-naphthyl)ethylenediamine produces a bluish-red color.¹⁰⁴ (cf. Vol. IV, p. 221). Penicillin does not take part in the reaction, and its decomposition products do not interfere. Therefore, the technic should also be applicable to other procaine samples. Diazotization of the procaine at 5-10° gives a stronger color than when carried out at room temperature. Up to 0.005 mg. of procaine per ml. may be measured.

The yellow color on reaction of procaine with vanillin in hydrochloric acid solution is sensitive to 0.05 mg. per ml.¹⁰⁵ A reaction with *cis*-aconitic anhydride applies to all compounds having a tertiary amino group. Those without that group do not interfere.¹⁰⁶

Procaine can be read at 290.5 m μ and pH 6 in the presence of phenylephrine.¹⁰⁷ The latter does not absorb under those conditions. Sulfanilamide interferes. Boric acid does not interfere. Adrenaline and zinc sulfate interfere only when present in larger quantities than usually prescribed with procaine.¹⁰⁸

Decomposition of procaine gives *p*-aminobenzoic acid. Extraction of an alkaline solution with chloroform leaves the *p*-aminobenzoic acid in the aqueous phase.¹⁰⁹ The latter, when diazotized and coupled with thymol, gives an orange-yellow solution for reading photometrically. The *p*-aminobenzoic acid may also be diazotized and coupled with 1-naphthylamine.¹¹⁰ This will detect 0.005 mg. of *p*-aminobenzoic acid. Coupling with dimethyl-1-naphthylamine has also been reported.¹¹¹ If 4-aminobenzoic acid is also present, the azo compound of procaine is extractable with chloroform for reading at 530 m μ . Procaine is read fluorescently at 345 m μ .

Sample—Injection solutions. Dilute a known volume of the sample with water so that a 5-ml. aliquot contains 0.5-1 mg. of procaine hydrochloride. Develop a 5-ml. aliquot with sodium 1,2-naphthoquinonesulfonate.

¹⁰⁴ M. G. Ashley and J. F. Lees, *J. Pharm. and Pharmacol.* **6**, 50-4 (1954).

¹⁰⁵ G. I. Luk'yanchikova and V. N. Bernshtein, *Sb. Nauchn. Dokl. Etarropol'sk. Kraevoe Old. Vses. Khim. Obshchestva im. D. L. Mendeleeva* **1**, 31-4 (1960).

¹⁰⁶ E. G. Feldmann and H. M. Kochler, *J. Dental Research* **39**, 313-19 (1960).

¹⁰⁷ B. Salvesen, *Medd. Norsk Farm. Selsk.* **24**, 185-97 (1962).

¹⁰⁸ N. I. Krikova, *Med. Prom.* **10**, 41-4 (1956).

¹⁰⁹ J. Richter, *Arzneimittel-Forsch.* **4**, 686-7 (1954).

¹¹⁰ W. Oelssner, *Pharmazie* **7**, 133-43 (1952).

¹¹¹ R. F. Banfi and R. W. de Wikenski, *Rev. Asoc. Bioquim. Argentina* **29**, 100-6 (1964).

Ointments. Dissolve a 1-gram sample with 150 ml. of ether or ethyl acetate. Add 30 ml. of 1:72 hydrochloric acid. Stir well and shake. Allow the layers to separate and collect the lower aqueous layer. Repeat extraction of the organic layer with two additional portions of hydrochloric acid and filter the combined aqueous acid extract. Dilute to 100 ml. with water and develop with sodium 1,2-naphthoquinine-4-sulfonate.

Veterinary food products. Procaine penicillin. To a weighed sample, add 35 ml. of water, 15 ml. of 0.4% sodium hydroxide solution and chloroform according to the chart in Table 26. Shake vigorously for 3 minutes

TABLE 26. DETAILS FOR DETERMINATION OF PROCAINE PENICILLIN

Formulation		Details of Method			
Class	Group	Weight of sample taken g.	Volume of chloroform and aliquot taken	Volume of 0.1N hydrochloric acid ml.	Cell size cm.
Premix (Limestone)	I	0.1	200 ml., 10-ml. aliquot	25	1.0
Premix (Kaolin)	I	0.1	200 ml., 10-ml. aliquot	25	1.0
Premix (Limestone)	I	0.2	50 ml., 10-ml. aliquot	25	1.0
Premix (Kaolin)	I	0.2	50 ml., 10-ml. aliquot	25	1.0
Premix	II	2.5	200 ml., 10-ml. aliquot	25	1.0
Concentrate	II	4.0	200 ml., 100-ml. aliquot	10	1.0
Concentrate	II	4.0	200 ml., 100-ml. aliquot	10	1.0
Foodstuff	II	10.0	200 ml., 100-ml. aliquot	10	1.0
Foodstuff	II	10.0	200 ml., 100-ml. aliquot	10	1.0
Foodstuff	II	10.0	200 ml., 100-ml. aliquot	10	2.0

and allow to separate. If a stable emulsion is formed, centrifuge at 2000 rpm until separation is completed. Filter the lower layer. Take an aliquot according to the chart and add the appropriate amount of 1:110 hydrochloric acid. Shake vigorously for 3 minutes and allow the layers to separate. Centrifuge the aqueous layer for 10 minutes at 2000 rpm. Cool 5 ml. of the upper layer to 5-10° and maintain at this temperature until the coupling agent is added. Develop by diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine.

Procedure—*By 1,2-naphthoquinone-4-sulfonate.* To prepare the acetate buffer, add 25 grams of sodium acetate trihydrate and 250 ml. of glacial acetic acid to water and dilute to a liter. Dilute a solution containing up to 3×10^{-6} mole of primary amine to 20 ml. with water and add exactly 3 ml. of 1:240 hydrochloric acid and 3 ml. of 6% sodium carbonate solution. Mix, and add 1 ml. of freshly prepared 1% sodium 1,2-naphthoquinone-4-sulfonate solution. Mix, and let stand in the dark for 90-120 minutes. Bleach excess reagent with 1 ml. of acetate buffer and 3 ml. of 4% sodium thiosulfate solution. Dilute to 50 ml. and read at $483 \text{ m}\mu$ 10-20 minutes after addition of thiosulfate.

Alternatively, dilute a sample containing up to 2 mg. of procaine hydrochloride per ml. to 100 ml. with water. Mix well and let stand for 5 minutes. To a 5-ml. aliquot, add 5 ml. of a 0.2% sodium 1,2-naphthoquinone-4-sulfonate solution and mix well. Read after 5 minutes at $490 \text{ m}\mu$ against a mixture of 5 ml. of reagent and 5 ml. of water.

By diazotization and coupling with N-(1-naphthyl)-ethylenediamine. To a 5-ml. aliquot of the sample containing up to 0.005 mg. of procaine per ml., cooled to $5-10^\circ$, add 1 ml. of 0.1% sodium nitrite solution and mix thoroughly. After 3 minutes, add 1 ml. of 0.5% ammonium sulfamate solution to remove free nitrous acid. Mix thoroughly and let stand for 2 minutes. Add 1 ml. of 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride solution and dilute to 10 ml. with water. Mix thoroughly and let stand for 20 minutes. Read at $605 \text{ m}\mu$.

In the ultraviolet simultaneously with tetracaine and cocaine. Dilute a sample containing procaine, tetracaine, and cocaine to 200 ml. with 0.12% sodium hydroxide solution. Add a 5-ml. aliquot to 10 ml. of 1.2% sodium hydroxide solution. Heat at 100° for 15 minutes. Cool, and dilute to 100 ml. with water. Read at $226 \text{ m}\mu$ for cocaine, $264 \text{ m}\mu$ for procaine, and $280 \text{ m}\mu$ for tetracaine, against 0.12% sodium hydroxide solution. Calculate as follows:

$$\begin{aligned}\text{Cocaine in ppm.} &= 42.24 A_{226} - 0.74 A_{264} - 12.00 A_{280} \\ \text{Procaine in ppm.} &= 2.49 A_{226} + 37.59 A_{264} - 24.42 A_{280} \\ \text{Tetracaine in ppm.} &= 1.06 A_{226} - 27.01 A_{264} + 36.02 A_{280}\end{aligned}$$

By nitrous acid. To a 0.07-0.13 ml. sample containing 0.05% of procaine, add 0.25 ml. of 1:110 hydrochloric acid and 0.5 ml. of 0.07% sodium nitrite solution. Dilute with water to 7-8 ml. and add 1 ml. of 0.4% so-

dium hydroxide solution. Dilute to 10 ml. with water. Compare with standards.

TETRACAINE, 2-DIMETHYLAMINOETHYL ESTER OF
p-BUTYLAMINOBENZOIC ACID

After the butyl of the amino group is released by bromine, the free amino group is diazotized and coupled with *N*-1-naphthylethylenediamine to produce an orange-red color¹¹² (cf. Vol. IV, p. 222). *p*-Aminobenzoic acid and adrenaline do not interfere. Tetracaine can be read directly at 311 m μ in the presence of phenylephrine,¹¹³ which does not absorb at that wave length.

Procedure—*By diazotizing and coupling with N-1-naphthylethylenediamine.* To a 1-ml. sample containing 0.05-0.2 mg. of tetracaine hydrochloride, add 1 ml. of 1:110 hydrochloric acid and 1 ml. of 0.5% bromine solution. After 15 minutes, add 1 ml. of 0.5% sodium nitrite solution and 1 ml. of 1:9 hydrochloric acid. After 3 minutes, add 1 ml. of 1% ammonium sulfamate solution to remove excess nitrous acid. After the bubbling stops, add 1 ml. of 0.2% *N*-1-naphthylethylenediamine-hydrochloride solution. Dilute to 25 ml. with 1:10 hydrochloric acid and read at 500 m μ .

In the ultraviolet. Simultaneously with cocaine and procaine. See procaine, page 407.

DIPHENYLAMINE

Nitrous acid is used as the oxidizing agent for the development of diphenylamine. It is applicable in powders containing dinitrotoluene and butyl phthalate¹¹⁴ (cf. Vol. IV, p. 228). An alternative oxidizing agent is dichromate in sulfuric acid.¹¹⁵ For a general reaction for diphenyl bases, see that subject, page 425.

Procedure—*Powders.* Dissolve a 2.5-gram sample in 100 ml. of methanol and 50 ml. of ethyl acetate. Add 120 ml. of water and collect the precipitate. Heat the precipitate with 100 ml. of methanol to 95° for 15 minutes and dilute with water to 500 ml.

¹¹² O. Hrdý and A. Slouf, *Českoslov. farm.* **5**, 21-4 (1956).

¹¹³ B. Salvesen, *Medd. Norsk Farm. Selsk.* **24**, 185-97 (1962).

¹¹⁴ Jean Barlot, *Mem. poudros* **36**, 199-207 (1954).

¹¹⁵ Joseph Grodzinski, *Bull. Research Council Israel* **7A**, 21-8 (1957).

To a 2-ml. aliquot of the sample solution, add 2 ml. of 1:3 sulfuric acid and 1 ml. of 10% sodium nitrite solution. Stir in a bath at 15°. When the temperature of the solution reaches 20°, place the tube in the colorimeter. Set the instrument at 100% transmittance with a 2-ml. portion of the sample diluted to 5 ml. with water and read at 575 m μ . For the absorption after reaction with chloranil in chloroform, see Table 22.

Propellants. Grind a 0.25-gram sample to 40 mesh and dry in a vacuum desiccator over silica gel for 24 hours. Extract the sample at 100° in a rubber extraction apparatus for 90 minutes with 60 ml. of 95% ethanol. The rate should be such that 1-2 drops per second fall into the thimble. Dilute the extract to 100 ml. with ethanol. To prepare the reagent, add 2 ml. of 1% potassium dichromate solution to 100 ml. of 31% sulfuric acid. To a 1 ml. aliquot of the sample, gradually add 9 ml. of reagent. Mix, and read at 575 m μ against a reagent blank 3-20 minutes after addition of the reagent.

N,N'-DIISOBUTYL-*p*-PHENYLENEDIAMINE

In acetic acid solution, N,N'-diisobutyl-*p*-phenylenediamine is determined with iodine in hexane.¹¹⁶ The procedure is sensitive to 0.002 mg. per ml. of sample.

Procedure—Gasoline. Dilute a 40-ml. sample to 100 ml. with hexane. To a 50-ml. aliquot, add 10 ml. of 10% acetic acid and shake well. Add 5-10 ml. of a solution of iodine in hexane and shake until the mixture turns pink to red. To 10 ml. of the aqueous layer, add 10 ml. of glacial acetic acid and shake well. Read at 550 m μ .

Anti-detonating mixtures. Dilute a 1-ml. sample to 100 ml. with hexane. Follow the procedure under "gasoline," starting with "To a 50-ml. aliquot . . ."

N,N-DIALKYLANILINES, DIPHENYLAMINES, AND CARBAZOLES

These classes of compounds react with 5-nitroisatin chloride.¹¹⁷ Wave lengths for absorbance are shown in Table 27. Aniline and *N*-methylaniline

¹¹⁶ M. Randi, *Atti Accad. Agiati* **210**, 91-95 (1961).

¹¹⁷ Eugene Sawicki, Thomas W. Stanley and Walter Elbert, *Mikrochim. Acta* **1961**, 505-11.

TABLE 27. WAVE LENGTHS FOR ABSORBANCE AFTER TREATMENT WITH 5-NITROISATIN CHLORIDE

<i>Amine</i>	<i>Wave lengths mμ</i>		
<i>N,N</i> -Dimethylaniline	575	615	665
<i>N</i> -Ethyl- <i>N</i> -methylaniline	575	615	666
<i>N</i> -Alkyl- <i>N</i> -methylaniline	575	615	666
<i>N</i> -Benzyl- <i>N</i> -methylaniline	580	620	672
<i>N,N</i> -Diethylaniline	575	615	665
<i>N,N</i> -Di- <i>n</i> -propylamine	575	619	671
<i>N,N</i> -Dibenzylaniline	581	621	675
<i>N,N</i> -Dimethyl- <i>m</i> -toluidine	550	595	693
Carbazole	580	675	—
<i>N</i> -Methylcarbazole	648	675	—
<i>N</i> -Ethylcarbazole	650	675	—
Diphenylamine	715	765	—
<i>N</i> -Methyldiphenylamine	575	622	674

line give weak bands around 580 $m\mu$. Other polynuclear aromatic amines react to some extent, but their spectra differ appreciably from that of the dialkylanilines. Phenol gives a band at 525 $m\mu$ with weak absorption at 665 $m\mu$. α -Naphthol gives a band at 587 $m\mu$. Indole gives a band at 563 $m\mu$. All of these are weaker than the materials reported on the table. Pyrene and chrysene give weak to negative tests.

The weaker the reagent, the less the final color. The rate of passage of hydrogen chloride gas must be roughly standardized, but the time of passage is more important.

Procedure—As a reagent, dissolve 1.3 grams of 5-nitroisatin in 70 ml. of hot anhydrous *o*-dichlorobenzene. Add 2.1 grams of phosphorous pentachloride and boil gently for 3 minutes. Cool and dilute to 100 ml. with anhydrous *o*-dichlorobenzene. To 5 ml. of a solution of the test substance in *o*-dichlorobenzene, add 5 ml. of the reagent. Pass dry hydrogen chloride through a calcium chloride tube into the mixture for 1 minute. Heat at 100° for 5 minutes and cool. Dilute to 50 ml. with the solvent and read at a wave length selected from Table 27.

p,p'-DIOCTYLDIPHENYLAMINE

The general reaction of primary and secondary aryl amines with furfural in the presence of sulfuric acid is applied to the determination of

p,p'-dioctyldiphenylamine in hydraulic fluids.¹¹⁸ Sulfuric acid causes a haze with hydraulic fluid samples containing derivatives of silicon, making filtering or centrifuging difficult. This is overcome by addition of Celite to the sample. Under the conditions of the procedure, phenyl-1-naphthylamine gives a red color, phenyl-2-naphthylamine an orange color, and *N,N'*-diphenylphenylenediamine a blue color. The presence of these amines can be detected by measuring at 450 m μ , at which the dioctyldiphenylamine-furfural reaction product has almost no absorption. Standards must be run along with the sample, since the reaction does not go to completion. Degradation products of the amine and the hydraulic fluids do not interfere. The procedure analyzes 2-10 mg. of amine per ml.

Procedure—*Hydraulic fluids.* To a 0.2-gram sample, add 40 ml. of 2-propanol and 10 ml. of 1:1 sulfuric acid. Mix cautiously. Add 0.5 gram of Celite 503 or 535, and filter through glass wool, discarding the first few milliliters of filtrate. Collect two 10-ml. filtrates. Alternatively, centrifuge for 15 minutes at 2000 rpm. To one 10-ml. portion, add 1 ml. of 2-propanol as a blank, and to the second 10-ml. portion, add 1 ml. of 10% furfural solution in 2-propanol. Read the sample against the blank at 385 m μ after 30 minutes.

m-PHENYLENEDIAMINE

m-Phenylenediamine is determinable in aniline by diazobenzene chloride.¹¹⁹ Aniline forms a diazoamino compound with the reagent, but *m*-phenylenediamine forms an azo dye. The *o*- and *p*- isomers and the corresponding toluidines do not interfere.

Procedure—Dissolve 2.5 grams of aniline in 5 ml. of concentrated hydrochloric acid and dilute to 250 ml. with water. Mix 5 ml. with 50 ml. of 20% sodium acetate solution. Add 1 ml. of a 0.14% solution of diazobenzene chloride in water. Stir for three minutes. Add 50 ml. of 1:4 sulfuric acid, and after 10 minutes, read at 460 m μ .

p-PHENYLENEDIAMINE

p-Phenylenediamine is determined in the presence of its meta and ortho isomers by ninhydrin in potassium cyanide solution¹²⁰ (cf. Vol. IV,

¹¹⁸ S. W. Nickles and S. H. Judd, *Anal. Chem.* **30**, 2002-4 (1958).

¹¹⁹ B. I. Kissin and E. N. Kurakin, *Zavod. Lab.* **30**, 538-9 (1964).

¹²⁰ Robert Suffis, Adele Levy and Donald Dean, *Anal. Chem.* **36**, 646-7 (1964).

p. 227). Follow the procedure under *p*-aminophenol, page 396, using a sample containing up to 1 mg. per ml., and read at 540 m μ . It is also read simultaneously with *p*-aminophenol and 2,5-diaminotoluene as the acetyl derivatives, page 395. For the absorption after reaction with chloranil in chloroform, see Table 22.

N,N'-DIPHENYL-*p*-PHENYLENEDIAMINE

In the presence of concentrated nitric acid, diphenyl-*p*-phenylenediamine forms a red color.¹²¹ The procedure is applicable to 0.005-0.035 mg. per 10 ml. of solution free from lipids. When lipids are present, the color concentrates around them and does not go into the acid solution. In this case, the color is developed by using acetone containing nitric acid as the solvent.¹²² Beer's law is followed for 0.008-0.01 mg. per 10 ml. in the presence of lipids and the color is stable for several minutes.

Benzoyl peroxide oxidizes *p*-phenylenediamine compounds to the corresponding *p*-quinonediamines.¹²³ In rubber samples, the *N,N'*-diphenyl-*p*-phenylenediamine is separated by paper chromatography. The spots are extracted with benzene and developed with benzoyl peroxide.

Procedure—*Feeds containing 0.025% of diphenyl-p-phenylenediamine.* To a 10-gram sample, add 50 ml. of acetone and heat for 20 minutes with occasional shaking at a temperature that allows the solution to boil gently. Cool, dilute to 250 ml. with acetone, and mix. Allow the larger particles to settle. Saponify a 5-ml. aliquot by addition of 3 ml. of 5% ethanolic potassium hydroxide solution to remove chlorophylls and other substances causing turbidity. After 30 minutes at room temperature, add 20 ml. of water, 1 gram of sodium sulfate, and 15 ml. of benzene. Stopper, and shake for 5 minutes. Centrifuge at a high speed for 10 minutes to break the emulsion. Evaporate a 5-ml. portion of the benzene extract under nitrogen without heat. Add 10 ml. of concentrated nitric acid. Mix, and read at 490 m μ within 3 minutes.

Depot fat. Dissolve a 10-15 gram rendered sample in 40-50 ml. of acetone by warming. Stopper, and freeze at -20°C. overnight or longer to fractionally precipitate the fat, leaving the diphenyl-*p*-phenylenedia-

¹²¹ R. H. Bunnell, *Poultry Sci.* **35**, 960-1 (1956).

¹²² W. Pudelskiewicz, L. M. Potter, L. D. Matterson and E. P. Singen, *Poultry Sci.* **35**, 959-60 (1956).

¹²³ J. W. H. Zipp, *Rec. trav. chim.* **76**, 317-20 (1957).

mine in solution. Filter the cold solution through a cold sintered-glass funnel containing dry ice chips. Rinse with cold acetone. Concentrate the filtrate to 35 ml. by boiling under a stream of nitrogen. Dilute to 50 ml. and evaporate an aliquot under nitrogen. Distil at 225° for 30 minutes at 10 microns pressure. Dissolve the distillate in acetone and evaporate to dryness under nitrogen. Take up the residue in 10 ml. of acetone. Add 4 drops of concentrated nitric acid and read after 1 minute at 520 m μ .

Egg yolks. Extract the sample with hot 95% ethanol for 18 hours in a Soxhlet extraction apparatus. Dilute with water and shake out the liquid with petroleum ether. Evaporate a known aliquot under nitrogen. Distil an aliquot of the extract following the procedure for depot fat, starting at "Distil at 225° for 30 minutes . . ." Alternatively, follow the entire freeze dry procedure as described under depot fat.

Rubber. Follow the procedure for extraction from rubber samples as under *N*-phenyl-1-naphthylamine (p. 424), starting with "Pulverize a 1-gram sample . . ." and ending with "Dry the chromatogram and scan . . ." The R_F value for *N,N'*-diphenyl-*p*-phenylenediamine is 0.56. Cut the spots from the chromatogram and extract with benzene. Add 1 ml. of 4% benzoyl peroxide solution in benzene and dilute to 25 ml. with benzene. Read at 450 m μ .

2,5-DIAMINOTOLUENE

It is determined as the diacetyl derivative. *p*-Aminophenol and *p*-phenylenediamine may be present and determined simultaneously (p. 385) (cf. Vol. IV, p. 229).

BENZIDINE, 4,4'-DIAMINOBIIPHENYL

A method for benzidine involves oxidation with permanganate in the presence of nitric acid.¹²⁴ The concentration of permanganate is critical. Hydrochloric acid does not interfere (cf. Vol. IV, p. 230).

Benzidine, diphenylene, *o*-toluidine and *o*-dianisidine are determined by tetrazotization and coupling with *N*-ethyl-1-naphthylamine in an ethanol medium.¹²⁵ Benzidine is read at 620 m μ and diphenylene is read at 545 m μ .

¹²⁴ Rip G. Rice and Earl J. John, *Anal. Chem.* **27**, 1630-1 (1955).

¹²⁵ V. Kratochvil, M. Matrka and J. Marhold, *Collection Czech. Chem. Commun.* **25**, 101-7 (1960).

The chloramine-T method is based on the formation of a yellow oxidation product when an acid solution of benzidine is treated with toluene-*p*-sulfonchloroamide.¹²⁶ The yellow product is extracted with chloroform. This is a general method for *p-p'*-diaminodiphenyl derivatives. Benzidine in the range of 0.001-0.05 mg. can be measured. 2,4-Diaminodiphenyl, aniline, α - and β -naphthylamines give no color.

A 2,3-hydroxynaphthoic acid coupling method can be used only when benzidine is the only unsulfonated primary aromatic amine present.¹²⁷ Other unsulfonated primary aromatic amines, such as aniline, give the reaction. The method depends on the tetrazotization of benzidine and the formation of a chloroform-soluble azo dye by coupling with the 2,3-hydroxynaphthoic acid. The dye is extracted from the acid solution with chloroform.

Sample—Atmosphere. *Absorption in 1:110 hydrochloric acid.* Pour 15 ml. of 1:110 hydrochloric acid into each of two Gage absorbers¹²⁸ and connect them in series. Connect the outlet tube of the second absorber to the inlet tube of a calibrated rotameter with rubber tubing. Connect the outlet tube of the rotameter through a T-piece, one arm of which carries a valve-controlled air leak, to a vacuum line with rubber tubing. Turn on the vacuum, note the time, and adjust the valve on the air leak so that the rate of flow is 5-10 liters per minute. After the end of the sampling period, note the time. Calculate in liters the volume of atmosphere sampled from the total sampling time and the constant rotameter level. Develop benzidine by the chloramine-T method.

Atmosphere. Collection on an exposed dish to determine settled benzidine. Expose a dish, 20 cm. in diameter, for a suitable time. Cover and remove to an uncontaminated area. Add 25 ml. of warm 1:110 hydrochloric acid and stir to dissolve the amine. Determine benzidine by the chloramine-T method.

Clothing. Over a 2-gram sample, distribute evenly 2 ml. of 11.2% potassium hydroxide solution in 95% ethanol. After 10 minutes, transfer the sample to a Soxhlet thimble. Rinse out the beaker with 50 ml. of ether and add the washing to the flask of the Soxhlet extraction apparatus. Add an additional 50 ml. of ether to the flask and place the thimble in

¹²⁶ L. T. Butt and N. Strafford, *J. Appl. Chem.* **6**, 525-39 (1956).

¹²⁷ *Ibid.*

¹²⁸ J. C. Gage, *J. Sci. Instr.* **29**, 409; 1952.

the apparatus. Extract the sample with ether for 2 hours. Cool, and extract the ether with 50-, 25-, and 25-ml. portions of 1:59 hydrochloric acid, shaking vigorously for 1 minute for each extraction. Extract the combined acid extracts with three 5-ml. portions of chloroform. If the third chloroform extract is appreciably colored, extract with additional 5-ml. portions of chloroform until no more color is extracted. Dilute the aqueous layer to 100 ml. Develop a 25-ml. aliquot by the 2,3-hydroxynaphthoic acid coupling method. If the amount of amine is more than 0.1 mg. per gram of cloth sample, add a further 2 ml. of potassium hydroxide to the sample and repeat the ether extraction for another 2 hours.

Miscellaneous deposits, residues, liquors, intermediates, and products in which hydroxy bodies, sulfonated primary amines, and unsulfonated primary aromatic amines may be present. Heat a 1-gram sample with 50 ml. of water to about 50° and stir well. Make the solution alkaline with 10.5% sodium carbonate solution, stirring until the solution is alkaline to thymol blue paper, a pH of approximately 9. Cool to room temperature and extract with 50-, 25-, and 25-ml. portions of ether. Wash the combined ether layers with three 5-ml. portions of water. Extract the ether solution with 50-, 25-, and 25-ml. portions of 1:59 hydrochloric acid. Extract the combined acid extracts with three 5-ml. portions of chloroform—more, if necessary—until no more color is extracted. Dilute the aqueous layer to 100 ml. and mix. Develop with 2,3-hydroxynaphthoic acid. In the presence of other primary aromatic amines, develop by the chloramine-T method.

Procedure—*By oxidation with permanganate.* Dilute a sample containing about 4.6 mg. of benzidine dihydrochloride corresponding to 0.2 ml. of a 2.3% solution and containing 3 ml. of concentrated hydrochloric acid per liter of solution with 900-950 ml. of water. Add 4 drops of concentrated nitric acid and wash down with water. Mix by swirling. Add 1 ml. of 0.4% potassium permanganate solution, wash down the sides of the flask with water, and swirl. Dilute to 1 liter with water and mix well. Read against water at exactly 8 minutes after addition of the permanganate solution.

By chloramine-T. The volume of the sample should be approximately 50 ml. and the acidity between *N* and 0.1 *N*, preferably 0.2 *N*. To the sample containing 0.001-0.05 mg. of benzidine in a separatory funnel with a short stem, add 1 ml. of 10% chloramine-T solution in cold water.

Work in weak diffuse light. After 5 minutes, add 5 ml. of chloroform and shake vigorously for 30 seconds. Remove any drops of water from the stem of the funnel with filter paper. Remove the chloroform layer and repeat the extraction with two 2-ml. portions of chloroform. If the third chloroform extract is not colorless, repeat the extractions, using a smaller aliquot of the original sample. Dilute the extracts to 10 ml. with chloroform and remove any water present with anhydrous sodium sulfate. Mix, and let stand in the dark until ready for use. Read at 600 $m\mu$ against chloroform. If the amount of benzidine found is 0.05 mg. or above, use a smaller aliquot of the sample, or dilute the solution with an equal volume of chloroform.

By 2,3-hydroxynaphthoic acid. Neutralize a 25-ml. sample containing up to 0.05 mg. of benzidine with 10.5% sodium carbonate solution until alkaline to Congo Red paper. Add 2 ml. of 1:10 hydrochloric acid and 2 grams of potassium bromide. Swirl gently to dissolve and cool in an ice-water bath. Add 0.4 ml. of 3.5% sodium nitrite solution. Stir well and let stand for 15 minutes. If a positive reaction is not obtained with starch-iodide paper, add a further 0.2 ml. of sodium nitrite solution and check after 15 minutes with starch-iodide paper. Add 1 ml. of 3% sulfamic acid solution and let stand for 5 minutes. Check that the reaction with starch-iodide paper is now negative. Add the tetrazotized solution to 0.5 ml. of 0.6% 2,3-hydroxynaphthoic acid in 10.5% sodium carbonate solution and 10 ml. of 10.5% sodium carbonate solution. Wash in the last traces of sample with water and let stand for 30 minutes. Add 1:1 sulfuric acid, dropwise, with stirring until the solution is acid to Congo Red paper. Transfer the coupled solution to a separatory funnel and ensure that all carbon dioxide has evolved by carefully shaking the funnel and releasing the pressure. Extract with five 5-ml. portions of chloroform. The last chloroform extract should be colorless. Dilute to 25 ml. with chloroform, if necessary, and read.

HYDRAZOBENZENE

Hydrazobenzene rearranged to benzidine is determined after diazotizing.¹²⁹ The method is then similar to but not identical with one given for benzidine.

Procedure—Dissolve the sample containing 1-5 mg. of hydrazobenzene in 20 ml. of ethanol and 10 ml. of concentrated hydrochloric acid.

¹²⁹ Miroslav Večeřa and Jaromir Petránek, *Chem. Listy* **48**, 1351-3 (1954).

After 90 minutes, add 4 ml. of 3.5% sodium nitrite solution. After 5 minutes, add 1 ml. of 3% sulfamic acid solution. After 2 minutes, add 2 ml. of 0.1% solution of *N*-1-naphthylethylenediamine hydrochloride. Dilute to 25 ml. and read after 90 minutes.

SALICYLAMIDE

N,N-Dimethyl-*p*-phenylenediamine is a reagent for salicylamide.¹³⁰ For direct reading in the presence of acetylsalicylic acid, caffeine, and phenacetin see page 417. This amide is also determined as indophenol, page 371.

Procedure—To a sample containing 0.005-0.05 mg. of salicylamide per ml., add 2 ml. of Kolthoff-Vieschhauwer buffer for pH 8.4 (Vol. I, p. 172) and 2 ml. of isobutanol. Shake vigorously with 0.5 ml. of 0.05% *N,N*-dimethyl-*p*-phenylenediamine solution and 0.6 ml. of 0.02% sodium hypochlorite solution for 5 minutes and let stand at room temperature for 5 minutes. Dilute the organic layer with 0.2 ml. of anhydrous ethanol and read at 650 m μ .

1-NAPHTHYLAMINE

The solution of the precipitate from 1-naphthylamine by sodium vanadate in ethanediol-isobutanol is suitable for direct reading.¹³¹ The solution follows Beer's law at 0.007-0.143 mg. of 1-naphthylamine in the presence of appreciable amounts of 2-naphthylamine. The color is stable for several hours. It is also diazotized and coupled with 2-naphthol. This color is stable for several days and obeys Beer's law over the range of 0.007-0.07 mg. per ml. But in this case, 2-naphthylamine interferes. To determine 1-naphthylamine in air, absorb it in a solution of diazotized aniline in 30:70 ethanol-acetic acid.¹³² The raspberry color is sensitive to 0.003 mg. per 3 ml. For the absorption after reaction with chloranil in chloroform, see Table 22.

Procedure—*By sodium vanadate.* To 0.05-2 ml. of about 0.014% solution, add 0.5 ml. of 3% sodium vanadate octadecahydrate solution.

¹³⁰ Konosuke Murai, *Arch. Prac. Pharm. Japan* **21**, 58-60 (1961).

¹³¹ F. M. Albert, E. Butucanu, M. Cupier, and M. Stoia, *Rev. Roumaine Chim.* **9**, 441-3 (1964); *Stud. Cercet. Chim.* **13**, 449-51 (1964).

¹³² D. N. Vaskevich and T. I. Sergeeva, *Gigiena i Sanit.* **21**, No. 3, 41-4 (1956).

Adjust to pH 4 with a few drops of 1:2 hydrochloric acid. Dilute to 10 ml. with 1:1 ethanediol-isobutanol. Shake for 15-30 minutes until the blue-green precipitate is dissolved. Read at 574 $m\mu$.

By chromic acid. To 0.05 ml. of 0.1% to 0.5 ml. of 0.01% solution of sample, add 1 ml. of a 0.1% solution of 2-naphthol in ethanol. Add 1 ml. of 10% aqueous solution of sodium nitrite. Add 2 drops of 1:2 hydrochloric acid and 5 ml. of dioxan. Dilute to 10 ml. with 95% ethanol and shake for 2-3 minutes to dissolve the red precipitate. Read at 533 $m\mu$.

2-NAPHTHYLAMINE

In yellow food dyes, amines are removed by acid extraction and β -naphthylamine is separated by column chromatography.¹³³ It is then read at 275 $m\mu$. Beer's law is followed for 0.002-0.022 mg. of β -naphthylamine per ml. An alternative after separation is to diazotize and couple with *N*-(1-naphthyl)-ethylenediamine.¹³⁴

A modification of the alkaline development method (Vol. IV, p. 236) involves steam distillation of the free amine from a suspension of the food dye in a saturated salt solution. The percentage recovery of the β -naphthylamine depends upon the amount of glass wool used and the weight of the dye sample. In the modification described, the sample size is decreased to 1 gram and 2 grams of glass wool are divided into 20 wads and soaked in water to increase the volume of water for the distillation and to prevent bumping. The method even as modified is not suitable for samples containing less than 800 ppm. of free β -naphthylamine.

β -Naphthylamine in α -naphthylamine is developed with furfural in an acetic acid medium.¹³⁵ The test is sensitive to 0.2% of β -naphthylamine. In industrial plants, β -naphthylamine is determined in the atmosphere and in miscellaneous deposits by coupling with 2,3-hydroxynaphthoic acid or by development with hypochlorite.¹³⁶ The 2,3-hydroxynaphthoic acid coupling method can be used only when β -naphthylamine is the only unsulfonated primary aromatic amine present.

An alternative is the red product of reaction with chromic acid.¹³⁷

¹³³ Walter D. Conway and Elizabeth J. Letheo, *Anal. Chem.* **32**, 838-41 (1960).

¹³⁴ B. Rassler and H. Schon, *Clin. Chem. Acta* **6**, 583-4 (1961).

¹³⁵ Giovanni Parravano, *Chim. e ind.* **41**, 30-3 (1959).

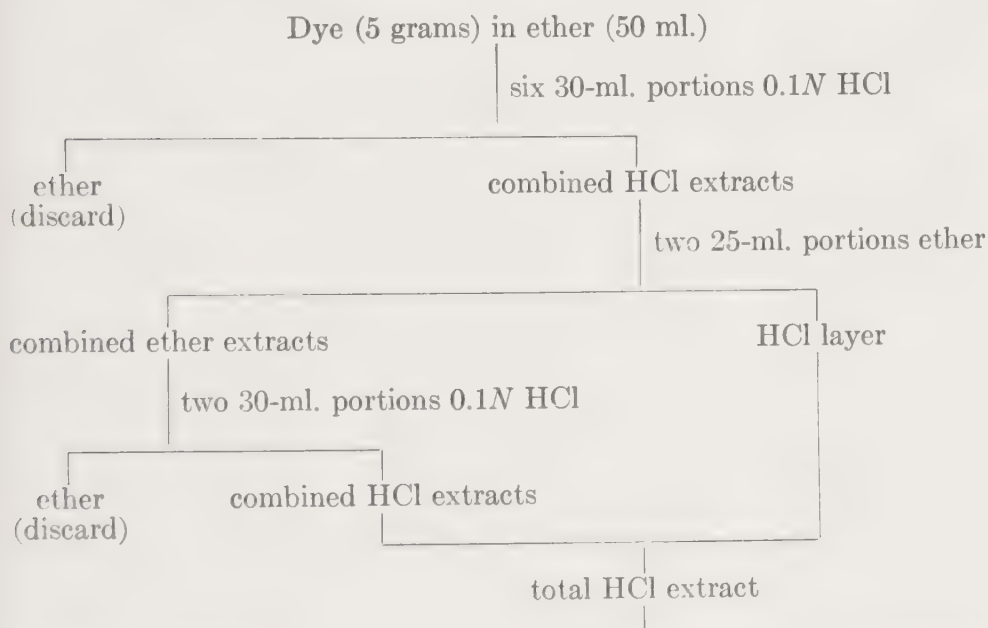
¹³⁶ L. T. Butt and N. Strafford, *J. Appl. Chem.* **6**, 525-39 (1956).

¹³⁷ F. M. Albert, E. Butuceanu, M. Cupfer and M. Stoia, *Rev. Roumaine Chim.* **9**, 441-3 (1964); *Stud. Cercet. Chim.* **13**, 449-51 (1964).

This is soluble in aniline-ethanediol and stable for 24 hours. Beer's law is followed over the range of 0.007-0.715 mg. in the presence of 5 times the amount of 1-naphthylamine.

In acid solution, β -naphthylamine forms a yellow color with sodium hypochlorite. The method is suitable for determination of amounts of β -naphthylamine ranging from 2.5 to 100% of the total primary aromatic amines—expressed as naphthylamine—present. Aniline interferes to a smaller extent than α -naphthylamine. Mixtures of aniline and β -naphthylamine with or without α -naphthylamine can be treated in the same way as mixtures of the α - and β -naphthylamines without serious error. Benzdine, tolidine, β -naphthol, and naphthylamine sulfonic acid interfere and must be removed. A general method outlined under dimethylaniline (p. 389) uses 4-azobenzenediazonium fluoborate.

Procedure—*Yellow AB or 1-phenylazo-2-naphthylamine or FD&C Yellow No. 3, and Yellow OB or 1-o-tolylazo-2-naphthylamine or FD&C Yellow No. 4.* Dissolve a 5-gram sample in 50 ml. of diethyl ether and extract according to the following scheme:



Carry out all extractions at 0° to minimize generation of free β -naphthylamine from the dye. Remove any ether present by means of a stream of air at room temperature. Dilute the total extract to 250 ml.

As one variant, to 5 ml. of the acid extract, add 1 ml. of 0.1% sodium nitrite solution to determine the total aromatic amines present. Mix, and after 3 minutes, add 1 ml. of 0.5% ammonium sulfamate solution. Mix, and after 2 minutes, add 1 ml. of 1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride solution. Dilute to 10 ml. with 1:110 hydrochloric acid and read after 40 minutes at 565 m μ . The color is at a maximum after 30 minutes and is stable for 2 hours.

For more accurate determination, concentrate the remaining 245 ml. of the acid extract *in vacuo* below 30°, using a rotating evaporator. The solvent system is prepared with 20 parts of 2-butanol, 10 parts of 2-propanol, 60 parts of hydrochloric acid-potassium chloride buffer at pH 1. Stir for several hours. The upper layer of the system is the organic or stationary phase. The lower layer is the aqueous or mobile phase. Dissolve the residue in the mobile phase so that 1 ml. contains approximately 0.125 mg. of β -naphthylamine per ml.

Soak Celite 545 overnight in 1:10 hydrochloric acid and wash with water until the washings are neutral to litmus. Dry at 120°. Coat with 2.5% of its weight of dimethyldichlorosilane by passing a stream of dry nitrogen first through the silane and then through a flask in which the resin is stirred. Wash the treated Celite with methanol and dry at 120°.

Slurry 15-20 grams of the Celite with 100 ml. of the aqueous phase and add 0.8 ml. of organic phase per gram of Celite. Evacuate the flask containing the slurry to 200-mm. pressure for a few seconds to remove entrapped air, and pour the slurry into a tube of 1.7 cm. inside diameter. After settling, pack the resin under 5-psi pressure for a few minutes, so that the finished column measures 1.7 \times 27.6 cm. for 20 grams of Celite. Adjust the flow rate to 18 ml. per hour by attaching a small-diameter polyethylene tube at the outlet of the column and by varying the height of the head of the solvent above the outlet tube. Measure the flow rate by inserting calibrated tubes at intervals in the collector rack. Pipet 2 ml. of the sample to the top of the column and rinse it into the column with a few ml. of the mobile phase. Collect 2 ml. fractions for the first 8 hours. Measure the light absorption of the effluent continuously at 254 m μ . Combine the tubes containing the β -naphthylamine fractions, rinse, and dilute to 25 or 50 ml. with the mobile phase. Read at 275 m μ .

By alkaline development. Follow the procedure outlined in Volume IV, page 236, under "Alkaline development," incorporating the following modifications:

1. Use a 1-gram dye sample.
2. Use 2 grams of glass wool divided into 20 wads. Soak in 200 ml. of water, and add the wool and the water to the distillation flask.
3. Distil until 125 ml. of distillate is obtained. Add and distil three separate 100 ml. portions of water to give a total of 425 ml. of distillate.

By furfural. In α -Naphthylamine. Distil a 30-ml. sample in a small vacuum still, to remove any color at the rate of 2-3 drops per second. Collect 25 ml. Dissolve 0.2 gram of the distillate and a sample of pure α -naphthylamine as a blank in 10 ml. of 95% ethanol. Transfer to amber glass flasks using 10 ml. of 95% ethanol as a rinse. Add 0.5 ml. of 20% acetic acid and 2 ml. of 5% furfural solution. Mix thoroughly and dilute to 25 ml. with 95% ethanol. Mix, and store in the dark at 20° for 90 minutes. Read at 530 m μ against the blank prepared from pure α -naphthylamine.

By 2,3-hydroxynaphthoic acid. Atmosphere. Follow either of the procedures under benzidine, page 415.

Miscellaneous samples. Follow the procedure under benzidine, page 415, and develop with hypochlorite.

Diazotization and coupling with 2,3-hydroxynaphthoic acid. Neutralize a 25-80 ml. sample containing 0.015-0.09 mg. of β -naphthylamine to Congo Red paper with 10.5% sodium bicarbonate solution. Add 2 ml. of 1:10 hydrochloric acid. Cool in ice water and add 0.4 ml. of 3.5% sodium nitrite solution. Stir well and let stand for 5 minutes. If a positive reaction is not obtained with starch iodide paper, add an additional 0.1 ml. of 3.5% sodium nitrite solution. Let stand for 5 minutes before re-testing. Pour the diazotized solution into a beaker containing 0.5 ml. of 0.6% 2,3-hydroxynaphthoic acid solution in 10.5% sodium carbonate solution and 10 ml. of 10.5% sodium carbonate solution. Wash in the last traces with water and let stand for 30 minutes. Extract with five 5-ml. portions of chloroform. The last extract should be colorless. Dilute the combined chloroform extracts to 25 ml., mix well, and read.

By sodium hypochlorite. In combination with α -naphthylamine. To prepare the sodium hypochlorite containing 0.1% of available chlorine, dilute technical sodium hypochlorite containing 10-14% of available

chlorine. Verify the strength of the reagent by titration with 1.7% sodium arsenite. The sample should contain 0.1 mg. of primary aromatic amine per ml. and the acidity should be 0.2 *N*. Dilute a 2.5-ml. sample to 20 ml. with 1:59 hydrochloric acid. Add 0.5 ml. of sodium hypochlorite reagent and dilute to 25 ml. with water. Let stand for 5 minutes and read against water at 600 m μ . This reading = *E*. From a calibration curve, read the weight of β -naphthylamine, which gives the same optical density as the sample. This reading = *M* μ g. From the calibration curve, read the optical density given by the α -naphthylamine in the aliquot taken. This reading = *F*. $E - F$ = the optical density given by the β -naphthylamine in the aliquot taken. From the β -naphthylamine calibration curve, read the weight of the β -naphthylamine, which gives an optical density = $E - F$. This reading = *N* μ g.

Calculate: β -naphthylamine in total naphthylamine taken is $(N \times 100) /$ (micrograms of primary aromatic amines taken, expressed as naphthylamine, mol. wt. 143).

If the β -naphthylamine is less than 25% of the total naphthyamines, dilute 5-, 7.5-, and 10-ml. aliquots of the sample each to 20 ml. with 1:59 hydrochloric acid. Add 0.5 ml. of sodium hypochlorite reagent for each 0.25 mg. of naphthyamines and dilute each to 25 ml. with water. Mix, and read after 5 minutes. In each case, read from a calibration curve the weight of β -naphthylamine required to give the same optical density as the sample. Correct the optical density for that contributed by α -naphthylamine and calculate % β -naphthylamine in the sample.

By chromic acid. Mix 0.5 ml. of 0.0014-0.00014% solution with 2 ml. of 1% chromic acid solution. Add 0.5 ml. of concentrated ammonium hydroxide and dilute to 10 ml. with 1:1 aniline-ethanediol. Shake for 2-3 minutes to dissolve the red precipitate. Read at 533 m μ .

N-PHENYL-1-NAPHTHYLAMINE

Addition of diazotized *p*-nitroaniline to *N*-phenyl-1-naphthylamine in acetone solution produces a blue-violet diazoamino compound¹³⁸ (cf. Vol. IV, p. 236). The intensity of the color increases during the first 10 minutes and then slowly decreases. Under the conditions of the technic, the color is constant for at least 20 minutes after the maximum color is reached. *N*-Phenyl-2-naphthylamine gives a reddish purple color with

¹³⁸ W. S. Levine and W. A. Marshall, *Anal. Chem.* **27**, 1019-22 (1955).

the reagent, which has an absorption maximum at 540 m μ . It is possible to distinguish between the colors visually. Other aromatic amines may interfere, as this is a basic reaction for aromatic amines. However, they are usually not present in oil samples. Beer's law is followed. The accuracy of the procedure is $\pm 10\%$.

In rubber samples, *N*-phenyl-1-naphthylamine is developed with diazotized sulfanilic acid.¹³⁹ Unvulcanized samples are extracted with ethanol and benzene, while vulcanized samples are extracted with benzene alone. The extract is chromatographed on acetylated filter paper. Successive extractions of a cyclohexane solution of an oil sample containing *N*-phenyl-1-naphthylamine with hydrochloric acid removes interfering amines. The naphthylamine is extracted with a mixture of concentrated hydrochloric acid and glacial acetic acid. The amine is recovered with a cyclohexane extraction and read in the ultraviolet at 338 m μ .¹⁴⁰ Beer's law is followed for 1-5 mg. per 100 ml. *N*-Phenyl-2-naphthylamine is also extracted by the acid mixture and can be distinguished from its isomer, since the 2-substituted amine has its peak at 309 m μ . It is possible to determine both isomers simultaneously by a series of simultaneous equations. Amines with no naphthalene ring do not interfere.

In chloroform the following naphthylamines develop color when exposed to ultraviolet light: 1-naphthylamine, violet color; *N*-phenyl-1-naphthylamine, blue; *N*-phenyl-2-naphthylamine, yellow; *N*-methyl-1-naphthylamine, blue; *N,N*-dimethyl-1-naphthylamine, violet to blue; diphenylamine, no color. The color intensity and absorption maximum varies with the irradiation time and solvent.¹⁴¹

To determine *N*-phenyl-1-naphthylamine in mineral oils, a known excess of chloroform is added to the oil and the color is formed by ultraviolet irradiation of the solution for 5-10 minutes. Beer's law is followed in the range of 10 to 100 ppm. in irradiated solution in chloroform at 640 m μ .

Procedure—*New and used oils. By diazotized p-nitroaniline.* To prepare the *p*-nitroaniline reagent, dissolve 1 gram in 50 ml. of 1:1 hydrochloric acid and dilute to 100 ml. Mix 5 ml. of *p*-nitroaniline solution with 1 ml. of 2.5% sodium nitrite solution and let it stand for 15 minutes before use.

Dissolve a 100-gram sample in 30 ml. of acetone with shaking. The

¹³⁹ J. W. H. Zijp, *Rec. trav. chim.* **76**, 313-6 (1957).

¹⁴⁰ Nicholas Feldman, *Anal. Chem.* **34**, 256-7 (1962).

¹⁴¹ Hans O. Spauschus, *ibid.* **33**, 1334-7 (1961).

amine is extracted into the acetone even when the oil is not completely dissolved. Carefully add 0.2 ml. of diazotized *p*-nitroaniline reagent, shake well for 30 seconds, and dilute to 50 ml. with acetone. Mix, and let stand at least 10 minutes. Read the sample at 555 $m\mu$. Prepare an oil blank for each type of oil blend to account for the influence of the color by using oil containing no *N*-phenyl-1-naphthylamine, and carry the blank through the procedure.

New and used oils. In the ultraviolet at 338 $m\mu$. Dissolve a weighed sample containing 10-30 mg. of *N*-phenyl-1-naphthylamine in 50 ml. of cyclohexane. Extract the solution with two 25-ml. portions of 1:10 hydrochloric acid and discard the extracts, which contain any interfering amines. If *N*-phenyl-1-naphthylamine is known to be the only naphthylamine present, this extraction may be omitted.

Extract the solution with two 10-ml. portions of a 3:1 mixture of glacial acetic acid and concentrated hydrochloric acid. Dilute the combined acid extracts to 150 ml. with water and alkalize with 5-50 ml. of 12% sodium hydroxide solution, adding an excess beyond neutralization. Extract the amine from the alkaline solution with two 125-ml. portions of cyclohexane and filter the extract through a small plug of cotton. Dilute the filtrate to 500 ml. with cyclohexane and read at 338 $m\mu$.

Rubber. Pulverize a 1-gram sample of unvulcanized rubber and extract in a Soxhlet apparatus with a 1:1 mixture of ethanol and benzene. Extract a 2-gram aged or vulcanized sample with benzene alone. Concentrate the extract to 10 ml. and chromatograph an aliquot on completely acetylated Whatman No. 1 filter paper by the ascending technique, using a 1:1 mixture of ethanol and benzene. Dry the chromatogram and scan under ultraviolet light to locate the spots. The R_F value for *N*-phenyl-1-naphthylamine is 0.64. Cut out the spots and extract for 90 minutes with 95% ethanol. The diazo reagent consists of 0.05% diazotized sulfanilic acid in a 1:1:2 mixture of ethanol-water-2:1 hydrochloric acid. To the extract, add 5 ml. of diazo reagent and dilute to 25 ml. with 95% ethanol. Read at 500 $m\mu$ for phenyl-1-naphthylamine and at 570 $m\mu$ for *N*-phenyl-2-naphthylamine.

N-PHENYL-2-NAPHTHYLAMINE

An aqueous solution of diazotized *p*-nitroaniline when added to an acetone solution of phenyl-2-naphthylamine produces an intense red

color.¹⁴² Since the color increases upon evaporation of the acetone, the reading is taken immediately. Beer's law is followed for 0.1-2 mg. per 100 ml. Compounds containing amino groups interfere. Phenyl-2-naphthylamine in rubber samples is also determined with diazotized sulfanilic acid as described under *N*-phenyl-1-naphthylamine.

Procedure—By diazotized *p*-nitroaniline. *Copolymers.* To prepare the coupling agent, dissolve 1 gram of *p*-nitroaniline in 50 ml. of 1:1 hydrochloric acid. To 25 ml., add 5 ml. of 2.5% sodium nitrite solution and let stand 15 minutes to react.

Extract a 1-gram sample with acetone for 3 hours. Rinse the apparatus with acetone, dilute the combined cooled acetone extracts, and rinse to 100 ml. with acetone. Dilute an aliquot containing 0.1-2 mg. of phenyl-2-naphthylamine to 100 ml. with acetone and add 1 ml. of coupling agent. Read immediately at 530 $m\mu$.

DIPHENYL BASES

Diphenyl bases in ethylene glycol are determined with *p*-dimethylaminobenzaldehyde solution in the presence of glacial acetic acid.¹⁴³ The following are the minimum concentrations of diphenyl bases detectable: benzidine 2×10^{-7} , 2,2'-dichlorobenzidine 2×10^{-6} , *o*-toluidine 1×10^{-7} , *o*-anisidine 1×10^{-7} , and 2,2'-dichloroanisidine 1×10^{-7} .

Sample—Air. Draw 100 liters of air per hour through absorption vessels containing 10 ml. of ethylene glycol. Mix 5 ml. of the absorbent solution with 45 ml. of glacial acetic acid and 0.25 ml. of 10% *p*-dimethylaminobenzaldehyde solution in acetic acid. Read the yellow color.

CYCLAINE, 1-CYCLOHEXYLAMINO-2-PROPYLBENZOATE

In alkaline solution, cyclaine rearranges to the amide. Upon hydrolysis, cyclaine yields the amine alcohol. All three components, cyclaine, the amino alcohol, and the cyclaine amide are separated, extracted with ethylene dichloride, and developed as the amino alcohol with methyl orange.¹⁴⁴ When the amino alcohol in ethylene dichloride is shaken with

¹⁴² P. Mensik and D. Broulik, *Rubber Chem. and Tech.* **29**, 647-50 (1956).

¹⁴³ P. Engelbertz and E. Babel, *Zentr. Arbeitsmed. u. Arbeitsschulz* **7**, 211-12 (1957).

¹⁴⁴ Vagel D. Wichehauss and Elizabeth R. Wyensky, *J. Lab. Clin. Med.* **49**, 651-6 (1957).

methyl orange and boric acid, salt formation occurs. The color is then developed by addition of alcoholic sulfuric acid. Beer's law is followed for 0.008-0.03 mg. of amino alcohol per ml.

Procedure—*Plasma, urine, or tissue extract.* To prepare the methyl orange reagent, wash a saturated methyl orange solution several times with ethylene dichloride. Before use, dilute the methyl orange solution with an equal volume of 3% boric acid solution.

Mix a 2-ml. sample with 2 ml. of 8% sodium hydroxide solution, and shake for 15 minutes with 6 ml. of ethylene dichloride to extract cyclaine, the amino alcohol liberated by cyclaine hydrolysis, and the cyclaine amide formed by the rearrangement of cyclaine in alkaline solution. Centrifuge for 10 minutes and aspirate the aqueous phase. Shake 4 ml. of the ethylene dichloride extract vigorously for 15 minutes with 4 ml. of 1:59 hydrochloric acid. Centrifuge for 10 minutes. The cyclaine and amino alcohol are extracted into the acidic phase and the amide remains in the organic phase. To 3 ml. of the acidic aqueous phase add 3 ml. of 4.8% sodium hydroxide solution and mix well. Develop a 2-ml. aliquot for the amino alcohol, as described below. Determine cyclaine in the remaining 3-ml. portion.

Place a small funnel in the top of the tube containing the 3-ml. portion for cyclaine determination, and heat at 100° for 1 hour to hydrolyze cyclaine. Cool, remove the funnel, and dilute to 3 ml. with water. Develop a 2-ml. aliquot for amino alcohol, as described below.

Take 3 ml. of the ethylene dichloride extract of the sample containing the cyclaine amide. Add 3 ml. of 4% sodium hydroxide solution and distil off all organic solvent at 100°. Place a small funnel in the top of the test tube and heat at 100° for 1 hour to hydrolyze the amide to amino alcohol. Cool, remove the funnel, and dilute to 3 ml. with water, if necessary. Develop the amino alcohol in a 2-ml. aliquot.

To develop the amino alcohol in each extract, add the 2-ml. alkaline aliquot to 20 ml. of ethylene dichloride. Stopper, and shake vigorously for 15 minutes. Centrifuge for 20-30 minutes and completely aspirate the aqueous phase. The amino alcohol is extracted into the organic phase. Add 15 ml. of the ethylene dichloride extract to 0.3 ml. of methyl orange reagent and shake vigorously for 25 minutes. Centrifuge for 20-30 minutes. Remove excess methyl orange solution completely by aspiration. Add 10 ml. of the ethylene dichloride solution to 2 ml. of 2:98 sulfuric acid-ethanol and read the color at 540 m μ after 10 minutes.

N-1-NAPHTHYLPHTHALAMIC ACID

Hydrolysis of the amide linkage of *N*-1-naphthylphthalamic acid yields 1-naphthylamine and sodium phthalate. The naphthylamine is steam-distilled to separate it from other interfering substances and coupled with diazotized sulfanilic acid to give a red azo dye.¹⁴⁵ Beer's law is followed.

Procedure—Homogenize a 250-ml. sample of vegetable tissue to obtain a representative sample. Use a portion containing not more than 0.02 mg. of the test acid. To prepare the sulfanilic acid, dissolve 1 gram in 100 ml. of 30% acetic acid by warming. For the diazonium reagent, mix equal parts of sulfanilic acid reagent and 0.12% sodium nitrite solution 2-5 minutes before use.

Wash a 2-10-gram sample into a hydrolysis flask, using 30 ml. of 30% sodium hydroxide solution. Swirl, connect the apparatus, and heat for 20 minutes or until about 15 ml. of distillate is collected. Add 5 ml. of glacial acetic acid to the distillate and ten drops of freshly prepared diazonium reagent. Dilute to 25 ml. and read the red dye after 30 minutes at 534 $m\mu$ against a reagent blank.

THIAMINE, ANEURINE, VITAMIN B₁

Oxidation of thiamine with potassium ferricyanide in an alkaline medium to thiochrome, followed by reading of its blue fluorescence in isobutanol in the ultraviolet is a common method for determination of thiamine¹⁴⁶ (Vol. IV, page 96). The alkali and ferricyanide are premixed, since direct addition of alkali to the sample destroys thiamine.¹⁴⁷ Thiamine in the sample is blocked with benzenesulfonyl chloride, the sample is developed, and the fluorescence measured is a measure of the interferences present.¹⁴⁸ Alternatively, the thiochrome is extracted with isobutanol. The isobutanol extract is then extracted with aqueous acid to separate the thiochrome in the aqueous layer from impurities retained

¹⁴⁵ Allen E. Smith and Gracie M. Stone, *Anal. Chem.* **25**, 1397-99 (1953).

¹⁴⁶ U. S. Pharmacopoeia XVI, 909-10 (1960).

¹⁴⁷ H. N. Haugen, *Scand. J. Clin. & Lab. Invest.* **12**, 253-60 (1960).

¹⁴⁸ H. N. Haugen, *Scand. J. Clin. & Lab. Invest.* **12**, 384-91 (1960); cf. K. Scharer and R. Preissner, *Landwirtsch. Forsch.* **7**, 36-45 (1954); C. Kawasaki and T. Itoh, *Bitamin* **13**, 391-3 (1957).

in the isobutanol. The separated aqueous layer is made alkaline and the thiochrome extracted into isobutanol. This may be repeated, if necessary, with the final reading in isobutanol.¹⁴⁹ Results in the presence of 1000 parts of sodium 4-aminosalicylate were low unless a large part of the latter was extracted with isobutanol before oxidation.¹⁵⁰

Urine samples are diluted to reduce the quenching effect of the reagent and washed with isobutanol to remove interferences. Interferences that fluoresce are destroyed by boiling for 15 minutes in an acidified solution at pH 2.¹⁵¹ There is no interference by vitamins A, C, E, B₆, and niacin under the conditions of test.¹⁵² In alkaline oxidation of thiamine by ferricyanide, the presence of 25% of methanol results in over 90% conversion at 0.0001-0.001 mg. per 8 ml. of reaction mixture.¹⁵³ Without it, 65-89% conversion is obtained. An alternative oxidizing agent is cyanogen bromide.¹⁵⁴

Thiamine, oxythiamine, and their phosphates are extracted from tissue and determined as thiochrome.¹⁵⁵ The tissue is extracted in the cold with 5% trichloroacetic acid solution. The extract is adjusted to pH 6.7-7 with 4% sodium hydroxide solution and sorbed on a carbon column. This is eluted with 10% propanol. The eluate is concentrated at 35° *in vacuo* and acidified with 0.8 ml. of 1:100 hydrochloric acid. Applied to a column of Dowex-1, the thiamine, oxythiamine, and their phosphates are eluted with water. The diphosphates are thereafter eluted with 1.36% sodium acetate trihydrate solution adjusted to pH 5.2. Incubating at 37° by phosphatase liberates the phosphate. Thiamine is determined as the thiochrome. The sum of thiamine and oxythiamine is determined by diazotized 4-aminoacetophenone for getting the latter by difference.

When blood is deproteinized by trichloroacetic acid, it does not remove penicillin, streptomycin, or sulfanilamides. In the thiochrome method, an intense green fluorescence results.¹⁵⁶ It can be avoided by deproteinizing with sodium sulfate. When protein-free biological extracts are oxidized

¹⁴⁹ Kunio Yagi, Toshikazu Tabata, Etsu Kotaki and Takeo Arakawa, *Vitamins* (Japan) **9**, 391-2 (1955); Penko Penkov, *Izvest. Otdel. biol. i Med. Nauki. Bulgar. Akad. Nauk.* **1958**, No. 4, 109-16; A. W. M. Indemans and H. E. J. Rademakers, *Pharm. Weekblad* **95**, 377-93 (1960).

¹⁵⁰ S. P. Sen and A. K. Sengupta, *Analyst* **89**, 558-9 (1964).

¹⁵¹ G. D. Eliseeva, *Vitamins, Akad. Nauk. Ukr. S.S.R.* **1**, 38-57 (1953).

¹⁵² L. Campanioli and M. Gianini, *Farmaco* (Pavia) *Ed. Prat.* **11**, 229-34 (1956).

¹⁵³ B. S. Wostmann and P. L. Knight, *Experimentia* **16**, 500-1 (1960).

¹⁵⁴ R. A. Bottomlay and S. Nobile, *J. Sci. Food Agric.* **13**, 546-550 (1962).

¹⁵⁵ L. DeGiuseppe and G. Rindi, *Z. Vitaminforsch.* **34**, 21-31 (1964).

¹⁵⁶ L. A. Stroganova and N. A. Kryukova, *Lab. Delo* **9** (12) 15-17 (1963).

with alkaline ferricyanide, the thiamine yields thiochrome, and pyriithiamine yields pyrichrome (2-methyl-4-amino-5-pyridylmethylpyridinium bromide).¹⁵⁷ The oxidation products are extracted with isoamyl alcohol. In the extract, thiochrome activated at 385 m μ fluoresces at 480 m μ . Pyrichrome in the extract activated at 410 m μ fluoresces at 480 m μ .¹⁵⁸ Each can be determined in the presence of the other. They can be sorbed on a column of powdered polyethylene, eluted with 0.4% sodium hydroxide solution in methanol, and read by fluorescence.

Thiamine and its esters are separable by paper chromatography, using 13:4:3 propanol-*M* acetate buffer-water.¹⁵⁹ Neopyriithiamine can be oxidized to thiochrome by ferric cyanide and extracted with isobutanol for fluorimetric estimation.¹⁶⁰ Interferences are extracted from acidified urine with isoamyl alcohol before making the urine alkaline for the thiochrome method.¹⁶¹

When a mixture of thiamine, pyridoxine, nicotinamide, riboflavine, and panthenol is passed through a column of decolorite, followed by a buffer for pH 2, the riboflavine is retained by the column. The panthenol does not interfere with the subsequent determination of thiamine.¹⁶² Thiamine and nicotinamide are also separable by paper chromatography, but it is more time-consuming.

Enzymatic digestion is necessary in analysis of bakery products because of the presence of cocarboxylase, the pyrophosphoric acid ester of thiamine. This pyrophosphoric group prevents extraction by isobutanol unless the acid group is removed. After acid hydrolysis, the cocarboxylase or the phosphoric acid ester of thiamine produced by acid treatment is removed by ion-exchange chromatography. For brewers' yeast, autolysis at pH 5.0 and 55° for 1-3 hours utilizes the phosphatase naturally present, converting cocarboxylase to thiamine.¹⁶³

The official fluorimetric method is modified for bread. The enzymatic digestion is reduced to 1 hour. Thiochrome in the blank is quenched with

¹⁵⁷ R. L. Airth and G. Elizabeth Foerster, *Anal. Biochem.* **3**, 383-95 (1962).

¹⁵⁸ G. Rindi and V. Perri, *Anal. Biochem.* **5**, 179-86 (1963).

¹⁵⁹ F. Olivo, F. Riva, D. Giarnieri and P. Fasella, *Ital. J. Biochem.* **11**, 118-21 (1962).

¹⁶⁰ G. Rindi and V. Perri, *Boll. Soc. Ital. Sper.* **35**, 2025-8 (1959); *Intern. Z. Vitaminforsch.* **32**, 398-405 (1962).

¹⁶¹ P. D. Starshov, *Lab. Delo* **8**, No. 6, 37-42 (1962).

¹⁶² D. K. Bardhan, R. N. Bhattacharya and S. K. Dutta, *Sci. Cult.* (Calcutta) **24**, 85-6 (1958); *J. Proc. Inst. Chemists (India)* **32**, 86-90 (1960).

¹⁶³ Atsushi Nakagawa, *Bull. Brewing Sci.* (Tokyo) **1**, 42-4 (1955); L. H. McRoberts, *J. Assoc. Offic. Agr. Chemists* **41**, 603-11 (1958).

hydrochloric acid. The method has been modified to use an acetate buffer for pH 4.8¹⁶⁴ for reading at 460 m μ .

Thiamine and riboflavine (Vol. IV, p. 296) may be determined in mixtures by fluorescence. Riboflavine emits a yellow-green fluorescence in neutral and in slightly acid solutions when excited by blue or ultraviolet light, and can be determined without prior chemical reaction. By controlling the pH of the solution containing thiochrome and riboflavine, both will fluoresce, and the amounts of each present can be determined by measuring the fluorescence at 450 and 530 m μ .¹⁶⁵ A standard deviation of 5% for each substance is attained for concentrations of 0.5-4 ppm.

A yellow color forms when thiamine is reacted with ninhydrin.¹⁶⁶ The formation of the colored compound is due to the thiazole portion of thiamine and not to the free amino groups.

When thiamine is treated with diazotized 6-aminothymol, an intense yellow color is produced that is soluble in an alkaline medium.¹⁶⁷ The procedure is less sensitive to interferences than is that with diazotized *p*-aminoacetophenone. The following do not interfere when present in concentrations up to 100 times that of thiamine: dextrose, lactose, maltose, sucrose, wheat flour, malt flour, casein, gelatin, peptone, urea, glycerophosphates, and salts of heavy metals. When present in concentrations up to 20 times the concentration of thiamine, the following do not interfere: riboflavine, nicotinic acid, nicotinamide, pyridoxine, pantothenic acid, *p*-aminobenzoic acid, creatine, xanthine, guanine, adenine, tryptophan, tyrosine, and histidine. Degradation products of thiamine give no color with the reagent. When the pH is below 5, there is loss of thiamine in the presence of metallic iron. Beer's law is followed up to 0.05 mg. per ml.

Diazotized *p*-aminoacetophenone (Vol. IV, p. 95) gives a purple-red color with thiamine.¹⁶⁸ This method is applicable to chocolate with extraction of the dye with xylene. The thiochrome method is not applicable due to interference by some natural constituents.¹⁶⁹ Diazotized sulfanilic

¹⁶⁴ A. W. Indemans and H. E. Rademakers, *Pharm. Weekblad* **95**, 377-93 (1960).

¹⁶⁵ W. E. Ohnesorge and L. B. Rogers, *Anal. Chem.* **28**, 1017-21 (1956).

¹⁶⁶ Yu. M. Ostrovskii and M. A. Gvozdeva, *Byull. Eksptl. Biol. i. Med.* **48**, 120-1 (1959).

¹⁶⁷ K. J. Hayden, *Analyst* **82**, 61-6 (1957).

¹⁶⁸ A. S. Vecher and O. B. Krayanskii, *Biokhimiya* **18**, 743-7 (1953); Shuntaro Ogawa and Seichi Nambu, *Bull. Natl. Hyg. Lab. Tokyo* No. **73**, 89-92 (1955); Z. Kleflin and K. Sumanovic, *Croat. Chem. Acta* **30**, 181-7 (1958); cf. O. S. Sherman and S. M. Kogan, *Trudy Vsesoyu. Nauch.-Issledovatel. Vitamin. Inst.* **4**, 230-4 (1953).

¹⁶⁹ W. Göpfert and P. Huppertz, *Deut.-Lebensm.-Rundschau* **50**, 120-1 (1954).

acid combined with formaldehyde is also used to develop thiamine.¹⁷⁰ Riboflavine and ascorbic acid interfere. Thiamine may also be coupled with diazonium salts of various sulfanilamides.¹⁷¹

Thiamine is precipitated at pH 4.5-5.5 by sodium phosphotungstate and determined nephelometrically.¹⁷² Thiamine can be determined as the reineckate at pH 4.5.¹⁷³ Below pH 6, it is precipitated as a direineckate. Above pH 8, it is mostly direineckate. In between, a mixture is precipitated.

Differential ultraviolet absorption at three wave lengths is effective in reading thiamine¹⁷⁴ in mixtures of vitamins B₁, B₆, and B₁₂. Thiamine has absorption maxima at 234 and 267 m μ at pH 7.0, 8.7, and 9.7, with the intensity increasing with pH. At pH 5 and 3, there was also progressive increase in absorption at those wave lengths.¹⁷⁵

Thiochrome in isobutanol can be read at 370 m μ or, after acidification with acetic acid, at 392 m μ .¹⁷⁶ The fluorescence of thiamine as thiochrome is eliminated by acidification with hydrochloric acid, thus giving a blank for correction.¹⁷⁷

Sample—*Dry and semidry nutritional materials containing no appreciable quantity of basic substances.*¹⁷⁸ Add 1:110 hydrochloric acid or 1:350 sulfuric acid equivalent to not less than 10 times the dry weight of the sample in grams. Stir. If lumping occurs, agitate vigorously and wash down the sides of the flask with 1:110 hydrochloric acid or 1:350 sulfuric acid. Digest for 30 minutes at 100° with frequent mixing, or autoclave the mixture at 121-123° for 30 minutes. Cool. If lumping occurs again, agitate vigorously. Dilute with the appropriate acid so that the thiamine content is 0.0002-0.005 mg. per ml.

Prepare on the day it is to be used a 10% aqueous solution of an

¹⁷⁰ Shuntaro Ogawa and Eiichi Hiraoka, *Vitamins* **4**, 359-61 (1951).

¹⁷¹ Aleksandra Smoczkieuczowa, *Acta Polon. Pharm.* **10**, 15-32 (1953).

¹⁷² V. N. Bernshtein, *Zhur. Anal. Khim.* **13**, 365-7 (1958).

¹⁷³ Denichi Ikeda, Fumiko Yaku, Hideko Yu and Naokazu Sakata, *Yakugaku Kenkyu* **33**, 26-35 (1961).

¹⁷⁴ G. Machek and F. Lorenz, *Sci. Pharm.* **31**, 17-26 (1963).

¹⁷⁵ N. P. Ivanov and G. K. Il'ich, *Lab. Delo* **8**, No. 2, 24-7 (1962).

¹⁷⁶ A. Gaudiano, *Rend. ist. super. sanita* **17**, 591-600 (1954); *Excerpta Med.*, Sect. II, **8**, 946 (1955).

¹⁷⁷ J. Janicki, E. Kamiski and Z. Bartold, *Nahrung* **6**, 423-9 (1962).

¹⁷⁸ Official Methods of Analysis of the Association of Official Agricultural Chemists, 8th Edition, Washington, D.C., 1955, pp. 819-823; Lewis H. McRoberts, *J. Assoc. Offic. Agric. Chemists* **40**, 843-52 (1957); *ibid.* **41**, 603-11 (1958).

enzyme preparation potent in diastatic and phosphorolytic activity. To prepare a bromocresol green indicator, dissolve 0.1 gram of the dye by triturating in a mortar with 2.8 ml. of 0.2% sodium hydroxide solution. Dilute to 200 ml. with water.

Dilute an aliquot of the sample containing 0.01-0.025 mg. of thiamine to 65 ml. with 1:350 sulfuric acid, and adjust the pH to 4-4.5 with 5 ml. of 27.5% sodium acetate solution, using the bromocresol green indicator. Add 5 ml. of the enzyme solution, mix, and incubate for 3 hours at 45-50°. Cool, dilute to 100 ml. with water, and filter through paper known not to adsorb thiamine.

To prepare the base-exchange silicate, cover 100-500 grams of 50-80 mesh grade with 2% acetic acid solution. Boil for 10-15 minutes, stirring constantly. Allow the mixture to settle and decant the supernatant liquid. Repeat with three additional portions of 3% acetic acid. Follow the same procedure, using three portions of hot 25% potassium chloride solution. Wash with boiling water until the wash gives no reaction for chloride. Dry at 100°. Alternatively, use ready-purified base-exchange silicate for thiochrome determinations.

The base-exchange tube has a glass reservoir at the upper end 50 mm. long and 25 mm. in diameter. This converges into the adsorption tube, which is 5-6 mm. in internal diameter and 140 mm. long. At the lower end of the tube, a capillary of 10 mm. is drawn, so that the rate of flow through the tube does not exceed 1 ml. per minute. Place a glass wool plug over the upper end of the capillary tube. Add a water suspension of 1-2 grams of base-exchange silicate, washing down all resin from the sides of the tube. Keep a layer of liquid above the resin to prevent air adsorption. To prepare acid potassium chloride, add 8.5 ml. of concentrated hydrochloric acid to 1 liter of 25% potassium chloride solution.

Pass an aliquot of the filtered solution containing approximately 0.05 mg. of thiamine through the base-exchange column. Wash the column with three 5-ml. portions of almost-boiling water. Do not allow the surface of the liquid to fall below the resin surface. Elute thiamine with five 4-4.5-ml. portions of almost-boiling acid potassium chloride solution. Cool, and dilute the eluate to 25 ml. with acid potassium chloride solution. Develop as thiochrome.

Dry and semidry nutritional materials containing large quantities of basic substances. Adjust the solution of the sample to pH 4 with dilute hydrochloric or sulfuric acids. Dilute with water so that the total volume in ml. is not less than 10 times the dry weight of the sample in grams.

Add 1 ml. of 3:1 hydrochloric acid or 1:3 sulfuric acid per 100 ml. of liquid, and follow the procedure for samples containing little basic material, starting with "Stir. If lumping occurs . . ."

Liquid nutritional materials. Adjust the pH to 4 with dilute hydrochloric or sulfuric acid, or by vigorous agitation with sodium hydroxide solution. Follow the procedure for semidry materials containing large quantities of basic substances, starting with "Add 1 ml. of 3:1 hydrochloric acid . . ."

Enriched flour. To prepare the thymol blue indicator, dissolve 0.1 gram with 4.3 ml. of 0.2% sodium hydroxide solution and dilute to 200 ml. with water. Select a suitable sample to contain about 0.0002 mg. of thiamine per ml. of final solution.

Mix the sample with water and add 5 grams of sodium chloride per 100 ml. of solution to aid separation. Add 15 ml. of 1:350 sulfuric acid per gram of sample in two portions with vigorous stirring. Heat for a total of 30 minutes at 100°, stirring for the first 5-8 minutes to keep solids in suspension during thickening. After 10 minutes of heating, test with thymol blue. The solution should be at pH 1-1.2, distinctly red. If the solution is not red, adjust with 1:35 sulfuric acid.

Note the amount of acid required and proceed with a new sample, adding the required amount of acid at the beginning of the analysis. Cool. Dilute with 1:350 sulfuric acid so that 1 ml. contains about 0.0002 mg. of thiamine. Centrifuge until a clear supernatant liquid is obtained. Alternatively, filter through paper known not to adsorb thiamine, or filter through a fritted-glass funnel. Discard the first 10% of filtrate.

As another technic, in place of digestion at 100°, follow the above procedure, omitting sodium chloride. Autoclave at 5 pounds pressure for 20 minutes at 108-109°, with a total heating time of not more than 35 minutes, including 5-10 minutes to attain the desired pressure and 5 minutes to reduce the pressure. Develop as thiochrome.

*Tablets.*¹⁷⁹ Weigh and powder 20 tablets. Mix a weighed quantity equivalent to 50 mg. of thiamine with 60 ml. of 1:13 hydrochloric acid. Let stand for 1 hour, with occasional shaking. Filter, wash the precipitate with the diluted hydrochloric acid, and dilute the filtrate and washings so that the final thiamine content is 0.02-0.04 mg. of thiamine per ml. Develop with 6-aminothymol.

¹⁷⁹ British Pharmacopoeia, London 1958, pp. 49-50.

Cereal enrichment materials. Shake a 1-gram sample with 100 ml. of water for 10 minutes. Filter rapidly, discarding the first 10-20 ml. of filtrate. Dilute an aliquot of the clear filtrate with 1:110 hydrochloric acid so that it contains 0.02-0.04 mg. of thiamine per ml.

Procedure—*As thiochrome.* As an oxidizing solution, dilute 4 ml. of 1% potassium ferricyanide solution to 100 ml. with 15% sodium hydroxide solution. Use within 4 hours. To prevent destruction, protect solutions from light during oxidation to thiochrome. Redistil isobutanol from an all-glass apparatus.

To each of 4 or more 40-ml. tubes, add 1.5 grams of sodium chloride or potassium chloride and 5 ml. of standard thiamine solution containing 0.001 mg. of thiamine per ml. in 0.1 N acid. For enriched flour samples, use 2.5 grams of potassium or sodium chloride. Add 3 ml. of oxidizing agent directly into one standard solution. Immediately add 13 ml. of isobutanol, stopper, and shake vigorously for 15 seconds. Treat one or more tubes of standard similarly, and treat the remaining tubes in the same manner, replacing oxidizing agent with 15% sodium hydroxide solution. Prepare four tubes of sample in the same way as the standards. Shake all the tubes for 2 minutes. Centrifuge at a low speed until a clear supernatant layer is obtained. Take a 10-ml. portion of the supernatant layer for reading. Measure the fluorescence of the isobutanol extract at $435\text{ m}\mu$,¹⁸⁰ with excitation at $365\text{ m}\mu$. Designate this as reading A. Measure the fluorescence of the sample treated with sodium hydroxide alone, and designate it as reading B. Measure the fluorescence of the standard oxidized, and designate as reading S. Measure the fluorescence of the standard treated with sodium hydroxide and designate it as reading D.

Micrograms of thiamine hydrochloride in a 5-ml. sample solution = $(A-B)/(S-D)$.

Urine. Mix 5 ml. of 40% sodium hydroxide solution and 0.6-1 ml. of 2% potassium ferricyanide solution. Add 10 ml. of a diluted sample over a 40-second interval with continuous shaking. Shake for an additional 50 seconds and add 2 drops of 3% hydrogen peroxide to stop oxidation. Add 10 ml. of a mixture of isobutanol and butanol, shake, and centrifuge. Read the fluorescence of the organic layer at $435\text{ m}\mu$ with excitation at $365\text{ m}\mu$.

¹⁸⁰ Kunio Yagi, Toshikazu Tabata, Etsu Kotaki and Takeo Arakawa, *Vitamin* (Japan) **9**, 391-2 (1955).

To prepare the blank, add 4 drops of 40% sodium hydroxide solution to a 10-ml. diluted sample. Add 1 drop of benzenesulfonyl chloride to block thiamine, shake, and follow the procedure for the sample, starting at the beginning.

Thiamine and pyriethiamine. To 3-5-ml. of sample solution containing 0.003-0.005 mg. of thiamine and 0.002-0.02 mg. of pyriethiamine, add 0.5 ml. of a 0.2% solution of potassium ferricyanide in 40% sodium hydroxide solution. As a hydrogen peroxide reagent, dilute 0.1 ml. of 30% solution to 10 ml. with water. After 4 minutes, add 0.1 ml. of this reagent. Pass through a 30 × 1.2-cm. column of polyethylene powder. Wash the column with 35 ml. of 0.4% sodium hydroxide solution. Elute the fluorescent band of oxidized perithiamine, which consists of 8-(hydroxyethyl)-2,9-dimethylperichromine, with 50 ml. of 0.4% sodium hydroxide solution in 5% methanol. Follow with 60 ml. of 0.4% sodium hydroxide solution in 8% methanol. Then elute the band of thiochrome with 30-50 ml. of 0.4% sodium hydroxide in 50% methanol. Read each by fluorescence, setting the instrument with 0.03 mg. of quinine sulfate per 100 ml. of 1:350 sulfuric acid.

Thiamine and riboflavin simultaneous in the ultraviolet. The apparatus consists of two Beckman DU monochromators, one as a monochromator for the exciting light M-1 and the other for fluorescence M-2. Allow the low-pressure mercury arc to warm up for 1 hour, and the phototube for 30 minutes, before measurements are made.

To a sample of not more than 8 ml. containing up to 0.1 mg. each of thiamine and riboflavin, add 2 ml. of 15% sodium hydroxide solution and 2 drops of 1% potassium ferricyanide solution. Shake for several seconds and let stand in the dark for 10 minutes. Add 2 ml. of 1:9 sulfuric acid, with shaking, to neutralize the base, and dilute to 25 ml. with a citric acid-dibasic sodium phosphate buffer. Mix, let stand in the dark for 10 minutes, and measure the fluorescence at 450 and 530 m μ . Adjust monochromator M-2 against standard samples of quinine containing 0.01 mg. quinine sulfate per ml. in 1:350 sulfuric acid.

Use the 365 m μ line for excitation and slit width settings of 2 and 1 mm. on the monochromators M-1 and M-2, respectively. The fluorescence intensity at 520 m μ of a 3-ppm. quinine solution is adjusted to read 35.0 on the per cent transmittance scale by adjusting the sensitivity knob of M-2. The fluorescence of a 0.3-ppm. quinine solution is measured with the same sensitivity settings by adjusting the per cent transmittance dial.

By p-aminoacetophenone. To prepare the diazo reagent, dilute 0.159 gram of *p*-aminoacetophenone in 2.25 ml. of concentrated hydrochloric acid to 25 ml. with water. Mix with 50 ml. of 4.5% aqueous sodium nitrite solution. Prepare a solution containing 5 grams of sodium hydroxide and 7.2 grams of sodium bicarbonate in 250 ml. of water.

Acidify a sample containing about 0.2 gram of thiamine hydrochloride in water with 20 ml. of 1:99 hydrochloric acid, and dilute to 250 ml. with water. To a 1-ml. aliquot of the sample, add 3 ml. of 0.3% phenol solution in ethanol, 0.5 ml. of diazo reagent, and 2 ml. of the alkaline solution. Cool on ice for 20 minutes. Add 7 ml. of xylene, shake, and let separate for 2 hours. Read the pink xylene layer at 525 $m\mu$ against xylene.

By 6-aminothymol. To prepare the reagent, dissolve 50 mg. of recrystallized 6-aminothymol hydrochloride in 50 ml. of 1:110 hydrochloric acid, and dilute to 100 ml. with water. To prepare thiamine stock solution, dissolve 100 mg. of thiamine in 100 ml. of 1:110 hydrochloric acid. As a working solution, dilute 10 ml. of the stock solution to 100 ml. with 1:110 hydrochloric acid. Prepare five tubes as follows:

1. 1 ml. of water.
2. 0.8 ml. of water and 0.2 ml. of the thiamine working standard containing 0.1 mg. of thiamine per ml.
3. 0.7 ml. of water and 0.3 ml. of the thiamine working standard.
4. 0.6 ml. of water and 0.4 ml. of the thiamine working standard.
5. 1 ml. of the sample solution.

Cool 0.5 ml. of 6-aminothymol reagent in ice water and add 2 ml. of 0.1% sodium nitrite solution. Mix, and let stand for 30-60 seconds. Add 5 ml. of 20% sodium hydroxide solution and dilute to 20 ml. Mix, and immediately add 1 ml. of this diazo reagent to each tube. After 5 minutes, dilute. If the sample solution is naturally colored or turbid, add a measured volume of a mixture of 90 ml. of toluene and 10 ml. of *n*-butanol to each tube. Shake to extract the color into the organic layer. Separate, and dry the solvent with anhydrous sodium sulfate. Read. Calculate thiamine as follows:

$$\text{Thiamine, micrograms per ml.} = 30 + 10 \times \frac{(\text{tube 5}) - (\text{tube 3})}{(\text{tube 4}) - (\text{tube 3})}$$

Tube 1 should not exceed 0.05.

By diazotized sulfanilic acid and formalin. To prepare the diazo reagent, dissolve 4.5 grams of *p*-sulfanilic acid in 45 ml. of 2:3 hydrochloric

acid and dilute to 500 ml. with water. Dissolve 50 grams of sodium nitrite in 500 ml. of water. Cool both solutions and mix 1.5 ml. of each. After a few minutes, add 6 ml. of the sodium nitrite solution and dilute to 50 ml. with cool water.

Shake 5 ml. of 2% sodium hydroxide solution with 0.4 ml. of cooled diazo reagent for 1 minute. Add 0.4 ml. of 35% formalin solution, shake, and add 1 ml. of sample containing up to 0.1 mg. of thiamine in hydrochloric acid at pH 4.5. Read at 494 $m\mu$ after 90-120 minutes.

Direct reading. Thiamine, pyridoxine, and nicotinamide. Read at 247 $m\mu$, 260 $m\mu$, and 290 $m\mu$. Then solve the simultaneous equations for thiamine, pyridoxine, and nicotinamide. Each is determinable with an accuracy of 6%.

o-BENZOYLTHIAMINE DISULFIDE

After enzymatic reduction to thiamine, this compound is determined as thiochrome.¹⁸¹ An alternative is to reduce with 4 ml. of 1% sodium thiosulfate solution at pH 4-5 as adjusted with 1:10 hydrochloric acid. Thereafter, incubate at 60° for 20 minutes.

Procedure—To 5 ml. of sample solution equivalent to 1-10 micrograms of thiamine, add 5 ml. of 0.1% cysteine hydrochloride solution. Adjust the pH to 8.4-9.0 with 4% sodium hydroxide solution. Add 2 ml. of Clark and Lubs buffer for pH 8.6 (Vol. I, p. 175). Incubate at 37° for 1 hour. To an aliquot equivalent to 2-20 micrograms of thiamine, add 2 ml. of 50% Taka-diestase, adjust to pH 4.5-5 and incubate for 2 hours at 45-50°. Treat the hydrolyzate with Permutit. Elute with 40 ml. of 25% potassium chloride in 1:10 hydrochloric acid. Oxidize with 5 ml. of 1% solution of cyanogen bromide. Make alkaline with 2 ml. of 30% sodium hydroxide solution. Extract with 10 ml. of isobutanol and read fluorometrically.

SEVIN, 1-NAPHTHYL-N-METHYLCARBAMATE

1-Naphthyl-N-methylcarbamate is hydrolyzed to 1-naphthol with alcoholic sodium hydroxide. Other 1-naphthol conjugates are hydrolyzed by refluxing with dilute hydrochloric acid. The free naphthol is de-

¹⁸¹ I. Umemi, Kiyoshi Harada, Yokko Hirao and Hiroko Hirao, *J. Vitaminol.* **8**, 220-7 (1962).

terminated directly in an acetic acid medium by reaction with *p*-nitrobenzenediazonium fluoborate.¹⁸² 1-Naphthol, naphthol conjugates, and Sevin are determined in the range of 0.002-0.01 mg. per ml.

Procedure—Poultry tissues. The apparent water content of Florasil is adjusted to 3.5% as follows. To two 50-ml. portions of methanol, add Karl Fischer reagent, water factor approximately 0.2, until the same permanent color is obtained in both solutions. Stopper, and use one portion as a blank. To the remaining methanol, add 1-2 grams of Florasil weighed to the nearest 0.1 mg. Stopper, and shake well for 30 seconds. Calculate the apparent water content of the Florasil and add sufficient water to raise the water content to 3.5%. Mix the Florasil continuously in a closed container by rotation or other method for 24 hours. Recheck the water content, which should be 3.5-3.8%.

The chromatography tube is 18 inches long, and 20 mm. in outside diameter, with a medium porosity fritted glass disk above the stopcock. Add the Florasil to the tube to a depth of 1 inch. Saturate the column with methylene chloride, which has been previously saturated with water.

To each of two 10-20-gram samples, add 200 ml. of methylene chloride and 50 grams of anhydrous sodium sulfate. Put each in a blender at high speed for 90 seconds and filter through a fluted filter paper. Transfer the residue and filter paper back to the blender, and extract with 170 ml. of methylene chloride. Filter the slurry and combine the extracts of each sample.

To develop 1-naphthol, extract the combined methylene chloride extracts of one sample with two 25-ml. portions of 2% sodium hydroxide solution. To the combined alkaline extracts, add 4 ml. of concentrated hydrochloric acid. Extract with one 25-ml. portion and one 20-ml. portion of methylene chloride and combine the extracts. Add 5 grams of sodium sulfate. Pass through the Florasil column. Use 40 ml. of water-saturated methylene chloride to rinse the containers, and pass this through the column. Reserve as the sample for 1-naphthol.

To develop Sevin, combine the methylene chloride extracts from the second sample in a 500-ml. flask and attach a three-bulb Snyder column. Evaporate the methylene chloride at 100° and dissolve the residue in 200 ml. of petroleum ether. Extract the petroleum ether solution with 25 and 15 ml. portions of acetonitrile. Evaporate the combined acetonitrile extracts at 100° and dissolve the residue in 25 ml. of methylene chloride.

¹⁸² D. P. Johnson, F. E. Critchfield and B. W. Arthur, *J. Agr. Food Chem.* **11**, 77-80 (1963); D. P. Johnson, *J. Assoc. Official Agr. Chemists* **46**, 234-7 (1963).

Pass the methylene chloride solution of the sample through the Florasil column and collect the eluate. Use 100 ml. of water-saturated methylene chloride to rinse the containers, and pour it through the column.

To prepare the *p*-nitrobenzenediamine fluoborate, dissolve 0.025 gram in 5 ml. of methanol and dilute to 25 ml. with acetic acid.

To each eluate, add 1 drop of diethylene glycol and evaporate the methylene chloride at 50-mm. pressure. Add 1 ml. of 0.4% sodium hydroxide solution in ethanol to the flasks containing the free naphthol and 2 ml. to the Sevin samples. Rotate so as to wet the entire inner surface and let stand for 2 minutes. Add 9 ml. of glacial acetic acid to the naphthol and 18 ml. to the Sevin samples. Add 1 ml. of 0.25% *p*-nitrobenzenediazonium fluoborate solution and let stand for 1 minute at 25°. Read at 475 m μ , using acetic acid to zero the instrument.

Egg yolks. Follow the procedure under poultry tissues, using the methylene chloride extract containing Sevin, ending with "Evaporate the combined acetonitrile extracts at 100° . . ." Add 5 ml. of 0.4% sodium hydroxide solution in ethanol and let stand for 3 minutes at room temperature. Transfer to a separatory funnel and rinse the flask with 25 ml. of 2% sodium hydroxide solution. Add 100 ml. of methylene chloride and shake well. Add the aqueous extract to a funnel containing 4 ml. of concentrated hydrochloric acid. Extract the methylene chloride layer with an additional 20 ml. of 2% sodium hydroxide solution and add the extract to the same funnel containing the hydrochloric acid. Extract with 25-ml. and 20-ml. portions of methylene chloride, and combine the methylene chloride extracts. Add 5 grams of anhydrous sodium sulfate and pass through the Florasil as described under poultry tissues, using only 40 ml. of methylene chloride rinse solution. Develop, and read the color as for Sevin.

Liver and gizzard tissue. Hydrolysis and extraction of conjugated naphthol. Blend a 10-gram sample with 40 ml. of water at high speed for 30 seconds. Add 10 ml. of concentrated hydrochloric acid, and reflux for 10 minutes, using a water-cooled condenser. Filter through fluted filter paper and mark the filtrate A. Transfer the residue and filter paper to the blender and blend for 30 seconds at high speed with 150 ml. of methylene chloride. Filter through fluted filter paper and mark the filtrate B. Repeat the extraction of the residue using 100 ml. of methylene chloride. Filter, and combine the filtrate with the filtrate marked B.

To filtrate A, add 25 ml. of water and extract with two 100-ml. por-

tions of methylene chloride, adding the extracts to filtrate B. Extract the combined filtrate B with 25-ml. and 20-ml. of 2% sodium hydroxide solution. Combine the sodium hydroxide extracts and add 4 ml. of concentrated hydrochloric acid. Extract with 25-ml. and 20-ml. of methylene chloride. Combine the extracts and add 5 grams of anhydrous sodium sulfate. Pass through the Florasil column and complete as for 1-naphthol in poultry tissues. Use 40 ml. of water-saturated methylene chloride for the transfer.

ANTHRIMIDE, DIANTHRIMIDE, 1,1'-IMINODIANTHRAQUINONE

In sulfuric acid concentrations over 90%, anthrimide reacts with boric acid to give an absorption in the blue region.¹⁸³ The technic below is applicable up to 0.2 mg. There is no interference from impurities such as 1-chloroanthraquinone or 1-aminoanthraquinone.

Procedure—Dissolve a sample of about 20 mg. in 100 ml. of concentrated sulfuric acid. Dilute part of this to 10 volumes with water. Heat 5 ml. of this, 1 ml. of 1% aqueous boric acid, and 5 ml. of concentrated sulfuric acid. The temperature should be such as to evaporate the water content to under 2% but not produce white acid fumes. Cool and read at 630 m μ .

CIPC, CHLORPROPHAM, ISOPROPYL N(3-CHLOROPHENYL) CARBAMATE

Chlorpropham is an herbicide that hydrolyzes to 3-chloroaniline.¹⁸⁴ For macerated crops, the chlorpropham is extracted with ethylene dichloride. The extract is hydrolyzed thereafter. The resulting 3-chloroaniline is steam distilled, diazotized, and developed with N-1-naphthyl-ethylenediamine. Milk may be stored under refrigeration for 2 days without adverse effects. Storage for 2 days at room temperature is required to obtain reproducible results with urine.

Procedure—*Milk*. To 100 grams of milk in a Kjeldahl flask, add 6-8 drops of Dow-Corning Antifoam A. Attach to a digestion and distillation assembly (Fig. 11) and cautiously add 100 ml. of 50% sodium hydroxide

¹⁸³ Eitaro Hongo, *Japan Analyst* **10**, 1200-3 (1961).

¹⁸⁴ L. N. Gard and J. L. Reynolds and C. E. Ferguson, Jr., *J. Agric. Food Chem.* **7**, 335-8 (1959); M. Montgomery and V. H. Freed, *ibid.* **7**, 617-18 (1959); L. N. Gard and C. E. Ferguson, Jr., *ibid.* **11**, 234-7 (1963).

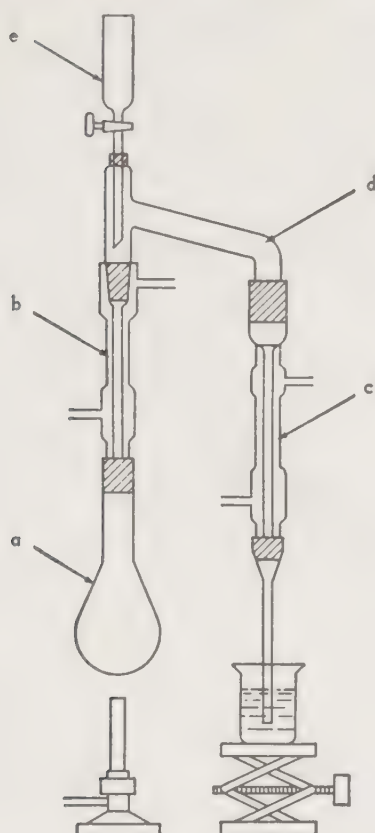


FIG. 11.

Hydrolysis and distillation apparatus: (a) 500-ml. Kjeldahl-type hydrolysis flask; (b, c) West-type condenser, 12 inches in length; (d) side-arm distilling head; (e) 125-ml. dropping funnel

solution. Rinse the funnel into the flask with 25 ml. of water. Mix by rocking. Reflux for 3 hours to hydrolyze the chlorpropham to 3-chloro-aniline. Discontinue the flow of water through the reflux condenser. Distil into 5 ml. of 3:2 hydrochloric acid until 30 ml. of distillate has been collected.

Add 0.2 grams of Celite filter aid to the distillate to remove some interfering materials. Mix, filter, and wash the filter until about 40 ml. is collected. Add 1 ml. of fresh 2% sodium nitrite solution and mix. After 20 minutes, add 1 ml. of fresh 10% sulfamic acid solution and mix. After 15 minutes add 5 ml. of 2% *N*-naphthylethylenediamine solution. Dilute to 50 ml. with 1:11 hydrochloric acid and mix. After 90 minutes read at 540 $m\mu$ against distilled water.

Urine. Add 0.5 ml. of formaldehyde solution per 450 ml. and age for 48 hours. Take a 200-gram sample and make slightly alkaline with a few pellets of sodium hydroxide. Shake vigorously for a minute with 150 ml. of ethanol and 75 ml. of petroleum ether. When separated, drain the lower layer into another extraction funnel and retain the ether phase in the initial funnel. Extract the aqueous phase twice more with 75-ml. portions of petroleum ether. Discard the aqueous phase and retain the petroleum ether extracts in the three funnels. Wash the petroleum ether extracts in the three successive funnels progressively with 3:1 ethanol-water, and discard the washings. Combine the ether phases in the hydrolysis and distillation flask. With a few glass beads to prevent bumping, distil to apparent dryness under reduced pressure.

Add 20 ml. of 1:1 sulfuric acid to the residue in the flask. Reflux for 90 minutes to hydrolyze the chlorpropham to 3-chloroaniline. Cool, and rinse the condenser into the flask with 100 ml. of water added through the top funnel. Complete as for milk, from "Discontinue the flow of water . . ."

CYCLOSERINE, *D*-4-AMINO-3-ISOXALIDONE

Cycloserine reacted with hydroxylamine is read at 520 $m\mu$.¹⁸⁵ Another method is to add phosphotungstic-phosphomolybdic acid reagent. It is then made alkaline with sodium carbonate and read at 770 $m\mu$.¹⁸⁶ At more than 0.002 mg. per ml., in slightly acid solution, it gives a readable blue color with sodium nitropentocyanoferrate.¹⁸⁷ Plasma or serum are clarified with tungstic acid. The cycloserine is read in an alkaline medium at 210 $m\mu$.¹⁸⁸

Procedure—To a sample containing 0.06-3 mg. of cycloserine, add 1 ml. of 6.6% hydroxylamine solution and heat at 60° for 10 minutes. Cool, and dilute to 3 ml. Add 1.4 ml. of saturated trichloroacetic acid solution and 0.6 ml. of 54% ferric chloride hexahydrate solution. Remove the protein precipitate and read at 520 $m\mu$ after 5-20 minutes.

¹⁸⁵ S. Schweet, *Biochim. et Biophys. Acta* **18**, 566 (1955); Hiromasa Ozaki, *Osaka Shiritsu Daigaku Igaku Zasshi* **9**, 3313-18 (1960).

¹⁸⁶ Toyozo Uno, Hiroyuki Yasuda and Takoshi Kondo, *Yakugaku Zasshi* **81**, 499-502 (1961).

¹⁸⁷ J. Viallier, Mme. N. Biot and R. M. Cayré, *Ann. biol. clin. (Paris)* **16**, 45-52 (1958).

¹⁸⁸ Janina Kolesińska, *Med. Doświadczalna i Mikrobiol.* **13**, 81-95 (1961).

7-HYDROXY-2-ACETAMINOFLUORENE

One of the metabolites of the carcinogen 2-acetylaminofluorene, and a probable metabolite of 2-aminofluorene, is 7-hydroxy-2-acetaminofluorene. It is determined by the yellow color formed when the hydrolyzed sample reacts with sodium nitrite.¹⁸⁹ The color is stable for 15 minutes to 1 hour. The maximum absorbance occurs at 400 m μ , but the yellow color is read at 450 m μ to eliminate some background absorption. Beer's law is followed for 0-0.025 mg., and the optimum concentration is 0.01-0.02 mg.

2,7-Diacetylaminofluorene and *N,N'*-diacetylbenzidine yield a yellow color with the reagent. 2-Acetylaminofluorene, 2-aminofluorene, 2-diacetylaminofluorene, 2,7-diacetylaminofluorene, 1-hydroxy-2-aminofluorene, and 3-hydroxy-2-aminofluorene interfere slightly, while negligible interference is observed with 2-monomethylaminofluorene, 2-dimethylaminofluorene, 1-diacetylaminofluorene, 4-acetylamino-4'-fluorobiphenyl and *p*-acetylaminobiphenyl.

Procedure—To a 0.5-ml. sample containing 0.01-0.02 mg. of 7-hydroxy-2-acetaminofluorene in glacial acetic acid, add 0.25 ml. of concentrated hydrochloric acid and 2 ml. of water. Heat at 100° for 1 hour to remove the acetyl group. Cool to 0° in ice and add 1 ml. of 0.2% sodium nitrite solution. Dilute to 6 ml. with water and read at 450 m μ 15-30 minutes after the addition of nitrite.

AMINOPYRINE, AMINOPHENAZONE

Aminopyrine is determined by diazotizing and coupling with phenyl-J-acid.¹⁹⁰ It is also determined with an ammonium vanadate reagent.¹⁹¹ Morphine and ascorbic acid interfere with the latter reagent. If phenobarbitone sodium is present, defer the reading for an hour. Another method of determination is with molybdophosphoric acid.¹⁹²

Procedure—By *phenyl-J-acid*. As a fresh reagent, dissolve 6 grams of sodium carbonate in 80 ml. of water and cool to 0°. Add 0.1 gram of phenyl-J-acid and dilute to 100 ml. with water. Store at 0°.

¹⁸⁹ Charlotte M. Dameron and Helen M. Dyer, *J. Natl. Cancer Inst.* **14**, 279-89 (1953).

¹⁹⁰ J. Gasparic, *Ceskosl. Farm.* **9**, 241-3 (1960).

¹⁹¹ K. Howorka and S. Beckert, *Pharm. Zentralh.* **102**, 715-18 (1963).

¹⁹² R. K. Mitra and G. K. Ray, *Indian J. Pharm.* **25**, 262-4 (1963).

Dissolve a sample containing about 4 mg. of aminopyrine in 50 ml. of 1:25 hydrochloric acid. Cool a 10-ml. aliquot to 0° and add 2 ml. of 2% sodium nitrite solution. Add 10 ml. of reagent to the diazotized sample. After 10-15 minutes, read at 490 m μ .

By ammonium vanadate. Mix a sample containing 5-50 mg. of aminopyrine with 20 ml. of 1:10 sulfuric acid. Add 20 ml. of a reagent containing 5.85 grams of ammonium vanadate and 10 ml. of concentrated ammonium hydroxide per liter. After 10 minutes, dilute to 50 ml. with water. Read at 590 m μ against a reagent blank.

By molybdophosphoric acid. Tablets. Reflux a sample containing about 250 mg. of aminopyrine with 25 ml. of 1:10 hydrochloric acid for an hour. Decant the solution and reflux the residue successively with 10 ml. and 5 ml. of 1:10 hydrochloric acid for 10 minutes. Wash the residue with about 40 ml. of water. Neutralize the extract and washings to pH 7, and dilute to 200 ml. Dilute an aliquot of this solution to contain about 0.2 mg. of aminopyrine per ml.

To a small aliquot, add 2.5 ml. of 0.4% sodium hydroxide solution and 2 ml. of 2% molybdophosphoric acid solution. Adjust the pH to 5.8-6 and heat at 80° for 90 minutes. Cool, and dilute to 10 ml. Read at 720 m μ against a reagent blank.

LIBRIUM, CHLORDIAZEPOXIDE

This is 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine-4-oxide. It is extracted with ether, rearranged in alkaline solution, and read by fluorescence. The method is sensitive to 0.25 microgram per ml.¹⁹³

Procedure—Plasma. Buffer the sample at pH 7.2. Extract the librium with ether. Evaporate the ether and take up the residue in water. Adjust the pH to 4.8 and hydrolyze to 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one-4-oxide. Expose to bright light to rearrange to a 4,5-epoxide. Make alkaline, activate at 380 m μ , and read the fluorescence at 480 m μ .

¹⁹³ Bernard A. Koechlin and L. D'Arconte, *Anal. Biochem.* **5**, 195-207 (1963).

DULCIN, *p*-PHENETOLCARBAMIDE

Dulcin is developed by oxidation with mercuric nitrate.¹⁹⁴

Procedure—To 4.5 ml. of sample solution, add 0.5 ml. of glacial acetic acid to clarify it. Add 0.5 ml. of a 2% solution of mercuric nitrate. Heat at 100° and cool. Add 0.5 ml. of a 1% solution of potassium persulfate. After 1 hour, add acetic acid and read at 550 m μ .

HYDRAZINOPHTHALAZINES

Reaction of 1-hydrazinophthalazine hydrochloride or sulfate with an acid solution of *p*-dimethylaminobenzaldehyde produces a yellow azine. The method is also applicable to 1,4-dihydrazinophthalazine sulfate.¹⁹⁵

Procedure—Dissolve a sample containing 10-70 mg. of 1,4-dihydrazinophthalazine sulfate in 20 ml. of water by heating to 100°. Dissolve a sample containing 60-140 mg. of 1-hydrazinophthalazine hydrochloride in 20 ml. of water. Dilute to 100 ml. with water.

To a 1-ml. aliquot, add 0.1 ml. of 4% *p*-dimethylaminobenzaldehyde solution in 1:16 sulfuric acid. Mix well, and let stand for 15 minutes for the sulfate and 10 minutes for the hydrochloride. Dilute to 50 ml. with water and read within 5 minutes.

ALLANTOIN OR ALUMINUM DIHYDROXYALLANTOINATE
OR ALUMINUM CHLOROHYDROXYALLANTOINATE

Allantoin is determinable in aqueous acid solution with copper tartrate and folin acid molybdate,¹⁹⁶ or phenylhydrazine and ferrieyanide.¹⁹⁷ The methods are applicable to allantoin, aluminum allantoinate, or aluminum chlorohydroxyallantoinate in cosmetics.¹⁹⁸

¹⁹⁴ Masuo Akagi and Setsuzo Tejima, *J. Pharm. Soc. Japan* 77, 1043-4 (1957).

¹⁹⁵ B. Wesley-Hadzija and F. Abaffy, *Croat. Chem. Acta* 30, 15-19 (1958).

¹⁹⁶ M. S. El Ridi, A. Aboul Magd and M. El Massy, *Proc. Pharm. Soc. Egypt. Sci. Ed.* 36, 71-7 (1954).

¹⁹⁷ E. Gordon Young and Catherine F. Conway, *J. Biol. Chem.* 142, 839-53 (1942).

¹⁹⁸ Sidney A. Katz, Richard Turse and S. B. Mecca, *J. Soc. Cos. Chem.* 15, 303-10 (1964).

Samples—Lipstick. Heat a 25 gram sample with 50 ml. of water to 100° and add 5 grams of ammonium sulfate. Mix in 5 grams of activated carbon. Filter while hot through a premoistened fluted filter. If still colored, repeat the treatment of the solution with activated carbon.

Suppositories. Suspend a 25-gram sample in 50 ml. of hot water. Add 5 ml. of concentrated hydrochloric acid and heat to 100°. Add 5 grams of ammonium sulfate. After 5-10 minutes at 100°, add 2 grams of activated carbon. Filter through a premoistened fluted filter.

Aerosol shave cream. To 15 grams of the shave cream, add 40 ml. of water and 5 grams of ammonium sulfate. Heat gradually to 100° with slow stirring. Dilute to 50 ml. with boiling water, and after 2 minutes, add 1 gram of activated carbon. Filter, while hot, on a premoistened fluted filter.

Cream, shampoo, after-shave lotion, or lotion. Mix a 25-gram sample with 50 ml. of water and 5 grams of ammonium sulfate. Heat at 100° for 2-5 minutes. Add 3 grams of activated carbon, and filter at once.

Talcum or compressed powders. Boil a 25-gram sample in 50 ml. of water with 5 grams of ammonium sulfate for several minutes. Add 2 grams of activated carbon and boil for 1 minute. Filter at once.

Procedure—By copper tartrate and Folin acid molybdate. As an ammoniacal copper reagent, dissolve 100 grams of ammonium sulfate in about 400 ml. of water. Filter, and to the filtrate add 100 ml. of 10% sodium hydroxide solution and 12 grams of sodium tartrate. Add 5 grams of cupric sulfate pentahydrate in aqueous solution. Dilute to 1 liter and store in a well-filled glass-stoppered amber bottle.

As Folin acid molybdate reagent, dissolve 300 grams of sodium molybdate in water and dilute to 1 liter. Add 2-3 drops of bromine and let stand overnight. Mix 500 ml. of the clear supernatant liquid with 225 ml. of 85% phosphoric acid. Add 150 ml. of cold 1:3 sulfuric acid. Drive off excess bromine by heating at 100° for 15 minutes. Cool, and add 75 ml. of glacial acetic acid. Dilute to 1 liter.

Extract the sample containing 20-100 mg. of allantoin with 25, 25, and 25 ml. of chloroform, and discard the extracts. If emulsions are troublesome, add 5 grams of ammonium sulfate to the aqueous layer. To the extracted aqueous layer, add 2 ml. of ammoniacal tartrate reagent.

After 10 minutes at 100° , cool and remove residual blue color by dropwise addition of 1:10 sulfuric acid. Add 2 ml. of Folin acid molybdate reagent. Dilute to 100 ml. After 15 minutes, read at $685\text{ m}\mu$.

By phenylhydrazine and ferricyanide. Extract the sample with 25, 25, and 25 ml. of chloroform, and discard the extracts. If necessary, to separate emulsions, add 5 grams of ammonium sulfate. To an appropriate aliquot containing 30-150 mg. of allantoin, add 10 ml. of 1:2 hydrochloric acid and heat at 100° for 3-5 minutes. Add 10 ml. of 1% phenylhydrazine hydrochloride solution while still hot. Cool, and add 0.5 ml. of 10% potassium ferricyanide solution. Mix, add 1 ml. of concentrated hydrochloric acid, and dilute to 100 ml. After 15 minutes, read at $530\text{ m}\mu$.

CHAPTER 6

AZO COMPOUNDS, NITROGEN-CONTAINING CYCLES, ETC.

SIGNIFICANT FLUORESCENT reactions applicable to this chapter are given in Table 28.¹

TABLE 28. SIGNIFICANT FLUORESCENT REACTIONS FOR AZO COMPOUNDS, NITROGEN-CONTAINING CYCLES, ETC.

Compound	pH	Excitation wave length	Emission wave length
		m μ	m μ
Amobarbital	14	265	410
Dromoran	1	275	320
Neocincophen	7	275, 345	455
Harmine	1	300, 365	400
Pentobarbital	13	265	440
Piperoxan	7	290	325
Phenobarbital	13	265	440
Pamaquin	13	300	370

1,2-Dimethyl-5-nitroimidazole, as a nitro compound, appears in this volume on page 24, and *methapyrilene*, on page 213. *Proline* and *hydroxyproline*, having nitrogen-containing cycles and being, as well, amino acids, are found on pages 296 and 299. *Cycloserine*, as an aromatic amine, and having a nitrogen-containing cycle, appears on page 442. The same criteria apply to *folic acid*, found on page 282.

Thiamine and *phenacetin* are aromatic amines with nitrogen-containing cycles, found on pages 427 and 399, respectively. *5-Aza-10-arsena-anthracene chloride* will appear in Volume IVAA. *Hydrazinophthalazine* and *1,4-dihydrazinophthalazine* are on page 445. A reaction of *dibutylamine* in acetone with *pyrocatechol* is also applicable to *piperidine* and *pyrrolidine*, on page 137. A method for *histidine* by *p-nitrobenzoyl chloride*, page 269, should be applicable to *imidazole* (cf. Vol. IV, page 286).

The *diethylamide of nicotinic acid* is determined by reaction with epi-

¹ Sidney Udenfriend, et al., *J. Pharmacol. Exptl. Therap.* 120, 26-32 (1957).

chlorohydrin.² The reaction is sensitive to 0.01 mg. per ml. *3-methyl-2-phenylmorpholine*, phenmatrazine, is read at 253.7 m μ as are many other sympathomimetic and stimulating amines.³

Uric acid appears at some length in Volume IIIA, pp. 410-17. After heat coagulation of 0.3 ml. of blood serum, uric acid is extracted with 3 ml. of water in 60 minutes at 37°. It is then read at 295 m μ , subject to error from drugs also absorbing in the ultraviolet. *Xanthine*, *hypoxanthine*, *uric acid*, and *6-mercaptopurine* at pH 1 have maxima at 265 m μ , 248 m μ , 284 m μ , and 326 m μ . The absorption of the first three is zero at 326 m μ . Thus, 6-mercaptopurine can be read in their presence. The others are read and solved by appropriate equations.⁴ For *nicotinic acid hydrazide* in a solution containing 2.5-40 mg., add 8 ml. of 4.8% cupric nitrate trihydrate solution and 5 ml. of acetone. Dilute to 25 ml. with water and read against a reagent blank.⁵ *Isonicotinamide*, *isonicotinic acid*, and *isonicotinylglycine* are determined by cyanogen bromide, page 488.

Meperidine, *pethidine hydrochloride*, *demerol*, shows a sharp peak at 257 m μ .⁶ *Dimethylimipramine* is read fluorimetrically in plasma.⁷ *5-Hydroxyindole acetic acid* is found in Volume IIIA, pp. 328-9. *Dibucaine hydrochloride*, *percuine*, is read fluorescently in 1:15 nitric acid.⁸ It is activated at 366 m μ and read at 420 m μ . The method determines 0.002-0.03 mg. per ml. within $\pm 1\%$. Procaine or tetracaine do not interfere, nor half the concentration of adrenaline.

Pentazole is precipitated as the reineckate in the presence of hydrochloric or nitric acid.⁹ The solution in acetone is read at 533 m μ . Precipitation as the reineckate, solution in acetone, and reading at 525 m μ is applicable to *phenindamine*, *meperidine* (*pethidine*), *mepazine* (*pecazine*), *thonzylamine*, and many other bases.¹⁰

3,5-Dicyano-1,2-dihydropyridine and its alkyl derivatives are read

² Andyzej Waksmundski and Henryk Romanowski, *Acta Polon. Pharm.* **11**, 205-9 (1954).

³ H. Thies and Z. Ozbilici, *Arch. Pharm., Berlin* **295**, 715-18 (1962).

⁴ A. Braschi and O. Cerri, *Boll. Chim.-farm.* **96**, 148-51 (1957).

⁵ S. Zommer and T. Lipiec, *Chemia analit.* **9**, 871-8 (1964).

⁶ H. J. van der Pol and R. F. Rekker, *Pharm. Weekblad* **96**, 41-8 (1961).

⁷ Celia M. Yates, A. Todrick and A. C. Tait, *J. Pharm. Pharmacol.* **15**, 432-9 (1963).

⁸ O. Hrdý and A. Slouf, *Ceskoslov. farm.* **1**, 71-5 (1952).

⁹ P. Spacu, Ilieana Albescu and Constanta Gheorghiu, *Acad. rep. populare Romane, Sesiile cercatarii chim.* **6**, 4565-72 (1958); Maria Smaga and Tadeusz Pelczar, *Dissertationes Pharm.* **12**, 275-80 (1960).

¹⁰ Kum-Tatt Lee, *J. Pharm. Pharmacol.* **12**, 666-76 (1960).

at 260 $m\mu$ in ethanol.¹¹ *Dextromoramide*, *yetryium*, and *palfium* are read in cyclohexane at 278 $m\mu$.¹²

Nicotyrine is determined in 1-10 microgram amounts after separation from other pyridine bases by chromatographing on alumina.¹³ It is then developed by cyanogen bromide and aniline. In the range of 1-3 mg. of 5,5-diphenylhydantoin per 100 ml., the solution in ethanol is read, converted to the sodium salt, and read again.¹⁴

A mixture of *pyrazinoic acid* and *pyrazinamide* absorbs with maxima at 268 and 269 $m\mu$. Addition of ferrous ammonium sulfate shifts a maximum to 460 $m\mu$ for a double salt of pyrazinoic acid.¹⁵ Each can then be read in the presence of the other.

2[(-*p*-Chlorobenzyl)-(2-dimethylaminoethyl)amino]pyridine, *halopyramine*, *Synopen*, is read in 95% ethanol at 245 $m\mu$ or 306 $m\mu$.¹⁶ Either wave length is accurate within $\pm 3\%$. In the presence of excess cyanoaceturia, 3-amino-5-pyrazolone is read at 298 $m\mu$.¹⁷ It also condenses with *p*-dimethylaminobenzaldehyde in 1:1 methanol-acetic acid and is then read at 490 $m\mu$.

The addition of ferric chloride in ethanol to a solution of 2-phenyl-3-hydroxy-4-quinolinecarboxylic acid or its sodium salt in pyridine gives a green color suitable for photometric estimation.¹⁸ The free acid shows an absorption maximum at 595 $m\mu$ and its sodium salt at 630 $m\mu$. Beer's law is followed for 0.01-0.1 mg. and the color is stable for 24 hours at 20°.

Many pyridine compounds are determined as molybdenum blue.¹⁹ One is *Ronicol*, 3-pyridinemethanol; another is *Trafuril*, tetrahydrofurfuryl nicotinate; and another, *Choligen*, the hydroxymethylamide of nicotinic acid. All are precipitated from acid solution by phosphomolybdic acid. The yellow precipitate is filtered and washed with 1:100 hydrochloric acid. Dissolve the precipitates in 0.4% sodium hydroxide solution and acidify with sulfuric acid containing ammonium molybdate. Ascorbic acid reduces the molybdenum in the precipitate to molybdenum blue. This is read at 700 $m\mu$.

¹¹ E. Janečková and J. Kuthan, *Coll. Czech. Chem. Commun.* **29**, 1495-8 (1964).

¹² Ernst Vidic, *Arch. Toxikol.* **19**, 254-68 (1961).

¹³ Monique de Clercq and René Trunaut, *Ann. pharm. franç.* **15**, 529-33 (1957).

¹⁴ I. Björge, J. Presthus and K. F. Stöa, *Medd. Norsk. Farm. Selskap* **19**, 17-22 (1957).

¹⁵ Takeshi Kimura and Norioki Goto, *Takamine Kenkyujo Nempo* **8**, 141-7 (1956).

¹⁶ R. Vasiliev and V. Scinteie-Pazarina, *Rev. chim. Bucharest* **14**, 236-7 (1963).

¹⁷ E. Swatek and J. Vacek, *Ceskoslov. farm.* **11**, 69-73 (1962).

¹⁸ Franco Pederzini and Pier Paolo Baracchi, *Boll. chim. farm.* **95**, 194-7 (1956).

¹⁹ Maria Smaga and Tadeusz Pelezar, *Dissertationes Pharm.* **12**, 275-80 (1960).

Dopamine is sorbed from urine at pH 8.5-9.0 on aluminum oxide at pH 8.5-9.0. Thereafter, it is eluted by dilute acetic acid, and is then oxidized with iodine and made alkaline with sodium hydroxide solution. This converts it to 5,6-hydroxyindole, which is read by activation at 320 $m\mu$ to give fluorescence at 375 $m\mu$.²⁰

Mecpyrium, 1-(2-propyl-4-amino-4-pyrimadylmethyl)-2-methylpyridinium chloride, is treated in alkaline solution with ferricyanide and naphthalenediol in methanol. This is read at 546 $m\mu$ and will determine 0.02 mg.²¹ *Pempidine*, 1,2,2,6,6-pentamethylpiperidine is determinable as the bismuth compound by the method described for benactyzine, page 133. It is accurate for 0.03-0.08 mg. per ml. when read at 500 $m\mu$.

Eradex, 2,3-quinoxalinedithiol cyclic trithiocarbonate is determined to 0.01 ppm.²² Extract the chopped sample with hexane and purify through an alumina column. Transform the product to the 2,3-diquinoxaline-ammonia complex, which is read at 530 $m\mu$. 1-Methyl-4(1)-quinoline, *echinopsine* and its salts are read by the red color at 0.0025 mg. per ml. or more on treatment with ferric chloride.²³ 3-Amino-5-pyrazolone in the presence of cyanoacetic hydrazide is read directly at 298 $m\mu$.²⁴ An alternative is to condense with *p*-dimethylaminobenzaldehyde in 50% acetic acid in methanol and read at 490 $m\mu$.

4-Dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one, *aminopyrine*, is read in ethanol at 270 $m\mu$.²⁵ Quinine hydrochloride has a maximum at 330 $m\mu$. Each is determinable in the presence of the other. *Aminopyrine* produces 4-aminoantipyrine by metabolism.²⁶ This condenses in ethanol with *p*-dimethylaminocinnamaldehyde in the presence of perchloric acid. The resulting 1-[*p*-dimethylaminophenyl]-3-[1-phenyl-2,3-dimethylaminopyrazolon-4-yl] trimethine perchlorate in chloroform has a maximum absorption at 511 $m\mu$. Similarly 4-aminoantipyrine condenses with *p*-dimethylaminophenylpentadenol to a pentamethine derivative absorbing at 590 $m\mu$.

γ -*Picoline oxalate* is successfully oxidized to isonicotinic acid in concentrated sulfuric acid containing selenium. The isonicotinic acid is then

²⁰ F. Bischoff and A. Torres, *Clin. Chem.* **8**, 370-7 (1962).

²¹ D. Monnier, J. Giacometti and R. Aries, *Mitt. Gebiete Lebensm. u. Hyg.* **51**, 187-95 (1960).

²² H. Tietz, M. F. Osman, H. Frehse and H. Niessen, *Pflanzenschutz-Nachr. "Bayer,"* **15**, 166-71 (1962-3).

²³ L. K. Sukhomut and A. S. Prozorovskii, *Apl'chnoe Delo* **8**, No. 3, 19-24 (1959).

²⁴ E. Svatek and J. Vacek, *Ceskoslov. farm.* **11**, 69-73 (1962).

²⁵ S. Bruno, *Il Farmaco* (Pavia) *Ed. Sci.* **10**, 922-4 (1955).

²⁶ Martin Strell and Siegfried Reindl, *Arzneimittel-Forsch.* **11**, 552-4 (1961).

read by ultraviolet absorption.²⁷ The ratio of the absorption before and after oxidation shows a minimum at 254 m μ and a maximum at 278 m μ .

o-Phenanthroline is read in ethanol at 323.5 m μ , or at 820 m μ , or in the presence of ferrous ion at 512 m μ .²⁸ 1,2-(2-Propyl-4-amino-4-pyrimidylmethyl)-2-methylpyridinium chloride, mephyrium, is oxidized in an alkaline methanolic medium in the presence of naphthalenediol. This determines 0.02 mg. of mephyrium at 546 m μ .

Phosphotungstic-phosphomolybdic acid forms the familiar molybdenum blue with many hydroxyindoles.²⁹ It is applicable to 4-, 5-, 6-, or 7-hydroxy-3-methylindole, 3-methylindolin-one, 5-hydroxyindole, 5-hydroxyindol-3-ylacetic acid, 5-hydroxytryptophan, 5,6-dihydroxy-1-methylindole and 3,5,6-trihydroxy-2-methylindole. After isolation of 1-80 micrograms of the compound in 2 ml. of dilute sodium hydroxide, add 1 ml. of reagent at one-third strength, 2 ml. of 20% sodium carbonate solution, and 8 ml. of water. After 1 hour, read at 730 m μ .

7-Methoxy-1-methyl-9-pyrid [3,4-*b*] indole, harmine is read fluorescently as the hydrochloride at 425 m μ .³⁰ Mixtures of *N*-methyl-4-phenyl-4-carbethoxypiperidine, meperidine, and benzyl-2-oxo-1-(2-piperidinocetyl)cyclocarboxylate, cetran, are read at two wave lengths in the ultraviolet.³¹

1-Diethylcarbamyl-4-methylpiperazine is extracted from blood and urine by ethylene dichloride.³² After addition of a 0.05% solution of bromothymol blue, this is centrifuged. The organic layer absorbs at 620 m μ if alkaline or 405 m μ if acid. When reacted with pyridine, 2,4-dichloro-6(*o*-chloroanilino)-*s*-triazine and related compounds absorb at 440 m μ if there is a chlorine in the ortho-, meta-, or para-position of the benzene ring.³³

To determine chloramphiramine,³⁴ first separate by steam distillation. Then pass an aliquot of the distillate through a column of Amberlite

²⁷ Harutado Negoro, *Ann. Rept. Takamine Labs.* **5**, 71-5 (1953).

²⁸ P. J. Seerest, J. A. Pawley and Claude A. Lucchesi, *Appl. Spectroscopy* **13**, 141-3 (1959).

²⁹ R. A. Heacock and M. E. Mahon, *Anal. Biochem.* **8**, 401-6 (1964).

³⁰ Kunio Yagi, Toshikazu Tabata, Etsu Kotaki, Tokeo Arakawa, *Vitamins (Japan)* **9**, 391-2 (1955).

³¹ G. Machek and F. Lorenz, *Sci. Pharm.* **31**, 17-26 (1963).

³² Hironori Fujimeki, *Nagasaki Igakkai Zasshi*, **30**, 1956-8 (1955).

³³ H. P. Burchfield and Eleanor E. Storrs, *Contribs. Boyer-Thompson Inst.* **18**, 319-30 (1956).

³⁴ Tetsuyasu Hatterri and Michinori Nishiumi, *Arch. Proc. Pharm. Japan* **21**, 9-14 (1961); Cf. C. Resta, *Farmaco (Pavia) Ed. prat.* **15**, 170-8 (1960).

CG-50 in the hydrogen form. Elute with 100 ml. of buffer for pH 2 and read at 264.5 $m\mu$. If *amidopyrine*, *methyllhesperidine*, and *methylephedrine* are present, separate them by elution with a buffer at pH 4.

To determine *pyridoxamine*, transaminate to pyridoxal with glyoxylic acid at pH 7.4 in the presence of aluminum ion. Then potassium cyanide yields a highly fluorescent pyridoxalecyanohydrin.³⁵ Results are linear over the range of 0.001-1 gamma per ml. *Iodoxuridine*, *2'-deoxy-5-iodouridine*, in aqueous solution is separated from decomposition products by chromatographing on a 30 \times 2-cm. column of Celite impregnated with 1:100 hydrochloric acid. Successive fractions eluted with 5:1 chloroform-butanol are *5-iodouracil*, *iodoxuridine*, *uracil*, and *2-deoxyuridine*.³⁶ Reading is at 292 and 310 $m\mu$ and estimation by their difference.

Promedol is treated with concentrated nitric acid and evaporated to dryness. The nitro compound is reduced to the amine with aluminum. This is then diazotized and coupled with quinoxol. The resulting red color is stable for 2 hours. The minimum detectable is 0.04 mg. per 100 ml.³⁷

To determine *simazin*, *2-chloro-4,6-bis(ethylamino)-s-triazine*, extract from plant tissue with chloroform. Extract acid-, alkali-, and water-soluble material from the chloroform. Evaporate the chloroform.³⁸ Hydrolyze the simazine with sulfuric acid to hydroxysimazine and read at 225, 240, and 255 $m\mu$. Not more than 0.015 mg. per ml. should be present.

2-Carboxy-4-isopropenyl-3-pyrrolidoneacetic acid, *kainic acid*, at 0.02-0.07 mg. per ml. conforms to Beer's law at 375 $m\mu$.³⁹ The color obtained by heating it with ninhydrin buffered to pH 7.0-7.1 for 30 minutes at 60° is read at 400 $m\mu$.⁴⁰

5-Acetamido-1,3,4-thiadiazole-2-thiol is read in 0.056% potassium hydroxide solution at 310 $m\mu$. Under the same conditions, *5-amino-1,3,4-*

³⁵ E. W. Toepler, M. M. Polansky and E. M. Hewston, *Anal. Biochem.* **2**, 463-9 (1961). D. E. Metzler, Joanne Olivard and Esmond E. Snell, *J. Am. Chem. Soc.* **76**, 644-8 (1954).

³⁶ C. A. Simpson and A. F. Zappala, *J. Pharm. Sci.* **53**, 1201-4 (1964); cf. Kiichiro Kakemi, Takaichi Arita and Mikiko Hashi, *J. Pharm. Soc. Japan* **84**, 1009-12 (1964).

³⁷ N. I. Krikova, *Uchenye Zapiski Pyatigorskii Farm. Inst.* **3**, 138-43 (1959).

³⁸ A. A. Petunova and E. E. Martinson, *Fiziol. Rast.* **10**, 729-31 (1963).

³⁹ Isao Satōda, Nobu Mitsumori and Shuzo Matsuo, *Yakugaku Kenkyū* **28**, 648-61 (1956).

⁴⁰ Shinzo Murakami, Tsumematsu Takemoto, Zensho Shimizu, Koji Daigo and Sasumu Hguchi, *J. Pharm. Soc. Japan* **74**, 85-7 (1954); cf. Masaharu Yamagishi, Hiroshi Morimoto, and Toshiro Matsuoka, *ibid.* **74**, 662-5 (1954); Masaharu Yamagishi, Hiroshi Morimoto, Toshiko Yoshida, and Toshiro Matsuoka, *ibid.* **73**, 1385 (1953).

thiadiazole-2-thiol absorbs at 293.5 $m\mu$.⁴¹ The maximum fluorescence of *cotarnine* is at 500-505 $m\mu$ in water, 480 $m\mu$ in dilute sodium hydroxide solution, 503 $m\mu$ in ethanol, and 470 $m\mu$ in ethanolic potassium hydroxide solution. In water, the intensity at pH 12.8 is suitable for reading. The fluorescence is linear for 0.1-1 microgram per ml.⁴² For *bendroflumethiazide* in tablets, extract with acetone. Evaporate the filtered extract to dryness and take up in absolute ethanol. Read at 270-273 $m\mu$.⁴³

Chlorpromazine can be extracted with sodium hydroxide solution, then extracted with ether. This, in turn, is extracted from the ether solution with 1:17 sulfuric acid by 1, 1, and 1 ml.⁴⁴ Heating the sulfuric acid extracts at 50-55° evaporates ether. Thereafter, addition of 2% ferric alum gives the color when diluted with 1:350 sulfuric acid. When chilled in ice, an equal volume of concentrated sulfuric is added. After cooling in the dark at room temperature, it is read at 530 $m\mu$. Many compounds interfere, including chlorpromazine sulfoxide, chlorpromazine-*N*-oxide, prechlorperazine, perphenazine, norchlorpromazine, thiopropazate, chlorprothixene, 2-hydroxypropazine, methoxypropazine, promazine, fluopromazine, fluophenazine, and thioridazine. Chlorpromazine forms a pink color by reaction with phosphotungstic acid, which is read at 530 $m\mu$.⁴⁵ 10-(Dialkylaminoalkyl)phenothiazines form a 1:1 complex with bromothymol blue. This can be extracted into chloroform for reading.⁴⁶

Pyrilamine maleate gives an intense blue with Folin-Ciocalteu reagent, which will determine in the range of 0.005-0.05 mg. per ml.⁴⁹ 2-Ethoxy-6,9-diaminoacridine lactate, *Rivanol* (Vol. IV, p. 312) oxidized with 1.9% sodium hypochlorite gives a red compound.⁵⁰ This can be read at 0.2-4 mg. to $\pm 2\%$. Acridine and atebrine interfere, but glucose, lactic acid, glycerol, urea, and boric acid do not. The absorption of 6-(4-carboxybutyl)purine at 299 $m\mu$ is used for its estimation.⁵¹ 6-Mercaptopurine can also be determined at 327 $m\mu$.

A method for *benzoquinolizines*, specifically applied to *Nitoman*,

⁴¹ J. Kráčmar and J. Vacek, *Českosl. Farm.* **9**, 497-502 (1960).

⁴² Y. Ichimura, *Japan Analyst* **8**, 557-61 (1959).

⁴³ Aluesio Marques Leal and Maria Beatriz S. Ramos Lopes, *Rev. Port. Farm.* **13**, 48-54 (1963).

⁴⁴ B. Godhelf, *Inst. J. Neuropharmacol.* **2**, 95-9 (1963).

⁴⁵ Sandor Farkas, *Magyar Kem Folyoirat* **64**, 209-11 (1958).

⁴⁶ Alexander Kotionis, *Arzneimittel-Forsch.* **11**, 108-10 (1961).

⁴⁹ M. Paz Castro and R. Rey Mendoza, *Galenica Acta* (Madrid) **14**, 285-91 (1961).

⁵⁰ C. Dragulescu and I. Florea, *Acad. Rep. Populare Romine, Baza Cercetari Stiint. Timisoara, Studii Cercetari Stiinte Chim.* **8**, 123-6 (1961).

⁵¹ F. Jančík, B. Káčáň, and B. Buděšínský, *Českoslov. farm.* **9**, 329-33 (1960).

2-dehydroemetine, and emetine calls for dehydration with mercuric acetate and reading the fluorescence.⁵² The method is sensitive to 0.02 mg. per 100 grams of sample. *Pentylentetrazole*, *Cardiazole*, gives a blue color with potassium chromate and hydrogen peroxide.⁵³ This is readable with an accuracy of $\pm 2\%$ in the presence of quinine, ephedrine, digynorm, foxglove leaves, or theophylline.

5-Aminoacridine hydrochloride emits fluorescently with a maximum at 467-465 m μ .⁵⁴ Alkaline hydrolysis of *hydrofluomethiazide* or *benzylhydrofluomethiazide* yields 4-amino-6(trifluoromethyl)-1,3-sulfamoylbenzene.⁵⁵ Coupled with chromotropic acid, it absorbs at 500 m μ . It is about 50% more sensitive when coupled with *N*-(1-naphthyl)ethylenediamine-dihydrochloride and read at 510 m μ .

Sulfamipyrine, *Melubrin*, can be hydrolyzed in aqueous solution to formaldehyde and aminoantipyrine. The latter is then determined by its reaction with dimethylaminobenzaldehyde.⁵⁶ 1-Phenyl-4-butyl-3,5-pyrazolidinedione (*monophenylbutazone*), and *phenylbutazone* are extracted from acid solution with ether. The extract is dried with anhydrous sodium sulfate and filtered. This is dried, taken up in ethanol, and read at 240 and 275 m μ , respectively.⁵⁷

Pyroglutamic acid is preferentially extracted from solid glutamic acid by water. This will detect 0.5% of pyroglutamic acid in the sample. It can be read at up to 0.002 mole per liter in saturated aqueous glutamic acid. The reading is at 233-236 m μ .⁵⁸ It is also developed with hydroxylamine and ferrous ion. This derivative of polyvinylpyrrolidone is preferably read spectrophotometrically, although a turbidimetric method is suitable for rapid control.⁵⁹ 6-(4-Carboxylutylthio)purine in 1:100 hydrochloric acid is read at 299 m μ .⁶⁰

The products of metabolism of antipyrine can be separated from urine and determined.⁶¹ To determine *rubazoic acid*, chromatograph on alumina

⁵² D. E. Schwartz and J. Rieder, *Clin. Chim. Acta* **6**, 453-63 (1961).

⁵³ A. Kolusheva and N. Ninlo, *Farmatsiya* (Bulgar.) **6**, No. 6, 19-23 (1956).

⁵⁴ J. G. Devi, M. L. Khorana, and M. R. Padhye, *Indian J. Pharm.* **15**, 3-5 (1953).

⁵⁵ J. Bermejo, *Galenica Acta* (Madrid) **14**, 255-64 (1961).

⁵⁶ Antal Végh, Gyorgy Szász, and Piroška Kertész, *Acta Pharm. Hung.* **31**, 49-54 (1961).

⁵⁷ M. Sahli and H. Ziegler, *Arch. Pharm. Chemi* **68**, 186-97 (1961).

⁵⁸ Guido Zuliani, Antonio Foffani and Ines Zanón, *Ricerca sci.* **27**, Suppl. A, *Polarografia* **3**, 88-93 (1957).

⁵⁹ K. Basu and B. N. Dutta, *J. Proc. Inst. Chem. India* **34**, 9-16 (1962).

⁶⁰ F. Jančík, B. Kakáč, and B. Buděšínský, *Českoslov. farm.* **9**, 329-33 (1960).

⁶¹ W. Hennig and H. Weiler, *Arzneimittel-Forsch.* **5**, 60-1 (1955).

and elute with ethanolic sodium hydroxide for reading. Determine 4-aminoantipyrine by adjusting the solution from which rubazoic acid has been removed to pH 9.8-10.2 with ammonia. Oxidize with ferricyanide, extract the color body with chloroform, and read. For 4-acetamidantipyrine, hydrolyze a sample of urine with sulfuric acid and determine the 4-aminoantipyrine formed. Moroxydine is read at around 0.005% in ethanol at 241 m μ .⁶²

4-(DIALKYLAMINO)-BENZENEDIAZONIUM COMPOUNDS

The absorbance at 380 m μ is a measure of the diazo nitrogen of stabilized diazonium compounds.⁶³ The method has been applied to 4-(diethylamino)benzenediazonium zinc chloride, 4-(dimethylamino)-, 4-(diethylamino)-, and 4-[ethyl(2-hydroxyethyl)amino]benzenediazonium salts. Beer's law is followed at 380 m μ for 1-10 ppm.

Procedure—To prepare the calibration curve, dilute a 5% 4-(diethylamino)benzenediazonium zinc chloride solution so that an aliquot gives 20-25 ml. of nitrogen. Dilute an aliquot so that about 1 mg. of diazo salt is contained in 100 ml. of water. Read at 380 m μ against water. Irradiate the solution of 4-(diethylamino)benzenediazonium zinc chloride to decompose it. Cool, and read the volume of nitrogen. Calculate the nitrometer results in moles of nitrogen per mg. and plot against the absorbancy.

Dissolve a 0.1-mg. sample and dilute to 100 ml. Dilute 1 ml. to 100 ml. Read immediately at 380 m μ . Read the moles of nitrogen from the calibration curve and calculate the per cent of diazonium compound:

$$\frac{\text{moles} \times \text{molecular weight} \times 100}{\text{weight of sample} \times \text{aliquot}} = \% \text{ diazonium compound}$$

1-(4-METHYLPHENYLAZO)-2-NAPHTHOL

D & C Red No. 35, which is 1-(2-nitro-4-methylphenylazo)-2-naphthol is sometimes contaminated by 1-(4-methylphenylazo)-2-naphthol. To separate these two compounds, their solubilities in ethanol are utilized. 1-(4-methylphenylazo)-2-naphthol being more soluble than the D & C

⁶² H. Siedlanowska, *Acta Polon. Pharm.* **21**, 33-40 (1964).

⁶³ Helen M. Rosenberger and Clarence J. Shoemaker, *Anal. Chem.* **31**, 204-6 (1959).

Red.⁶⁴ Both naphthols after separation are analyzed as a two-component mixture.

Procedure—*D & C Red No. 35*. Heat a 200-mg. sample with 75 ml. of chloroform at 100° until dissolved. Add 200 ml. of 95% ethanol and remove from the heat. Cool to 0-5° in ice. Filter the precipitated *D & C Red* through a retentive fluted filter and reserve the filtrate containing the 1-(4-methylphenylazo)-2-naphthol. Place the drained filter paper in the original flask and macerate with a glass rod. Add 75 ml. of chloroform and repeat the procedure of heating, precipitation with ethanol, and filtering. Evaporate the combined filtrates to 50 ml. at 100°, using a gentle air stream to speed the process. Cool to room temperature. Transfer, and rinse the container with several small portions of 95% ethanol. Dilute the solution and washings to 100 ml. with 95% ethanol. Filter through a retentive filter. Evaporate 50 ml. of the filtrate to dryness and dissolve the residue in chloroform. Dilute to 100 ml. with chloroform. Read at 420 and 512 $m\mu$. Calculate as follows:

$$aX + bY = A_{s(420)}$$

$$cX + dY = A_{s(512)}$$

in which

a = absorbancy per mg. per liter of 1-(4-methylphenylazo)-2-naphthol at 420 $m\mu$

b = absorbancy per mg. per liter of *D&C Red No. 35* at 420 $m\mu$

c = absorbancy per mg. per liter of 1-(4-methylphenylazo)-2-naphthol at 512 $m\mu$

d = absorbancy per mg. per liter of *D&C Red No. 35* at 512 $m\mu$

$A_{s(420)}$ = absorbancy of the sample at 420 $m\mu$

$A_{s(512)}$ = absorbancy of the sample at 512 $m\mu$

X = concentration of 1-(4-methylphenylazo)-2-naphthol in mg. per liter

Y = concentration of *D&C Red No. 35* in mg. per liter

PYRROLE

Pyrrrole forms a blue color upon reaction with isatin in an ethanolic medium at room temperature.⁶⁵ The maximum is at 500-520 $m\mu$ (Vol.

⁶⁴ Charles Grachen and Lee S. Harrow, *J. Assoc. Offic. Agr. Chemists* **35**, 754-7 (1952).

⁶⁵ Fritz Fromm, Rita Wilhelm and Ellen Jane McGrady, *Proc. Penn. Acad. Sci.* **30**, 108-10 (1956).

IV, pp. 241-2). The reaction is given only by pyrroles with at least one free α -position. Pyrroline, pyrrolidine, pyridine, indole, tryptophan, carbazole, and thiophene do not react. Beer's law is followed for up to 40 mg. of pyrrole per liter.

Procedure—Mix the sample of pyrrole in 0.5% acetic acid solution with 0.2 ml. of 0.5% isatin solution in glacial acetic acid. Add 1 ml. of concentrated hydrochloric acid and dilute to 10 ml. with 95% ethanol. Reflux for 10 minutes, cool to room temperature, and readjust the volume to 10 ml. with 95% ethanol. Read at 520 $m\mu$ against a reagent blank.

INDOLE, BENZAPYRROLE

The reaction of indole with ferric chloride in sulfuric acid at 100° produces a color suitable for reading.⁶⁶ Nitrous acid produces a red color with an aqueous solution of indole.⁶⁷ 3-Methylindole does not react alone, but when present with indole it interferes. Fractionation removes interference from homologs of indole. Hydrocarbons, aldehydes, ammonia and hydrogen sulfide do not interfere.⁶⁸

Indole in the presence of anthranilic acid can be developed in 0.5 *N* acid by *p*-dimethylaminobenzaldehyde and read at 550 $m\mu$ without interference, page 384. It can be read by the same reagent in the presence of tryptophan and anthranilic acid, page 267. Indole is also determined by condensing with the corresponding cinnamyl compound.⁶⁹ The latter is 2.2 times as sensitive and will determine 0.001 mg. in a 20-ml. sample.⁷⁰ There is no interference in the reaction with *p*-dimethylaminobenzaldehyde (Vol. IV, pp. 288-90) by gramine, tryptamine, tryptophan, indol-3-ylacetic acid, *N*-glycyltryptophan, 5-hydroxytryptamine, and 5-hydroxy-*N,N*-dimethyltryptamine. Tryptophan causes rapid fading of the color. Urea and anthranilic acid lessen the intensity of color developed. Riboflavin shifts the color maximum to 575 $m\mu$ and enhances the color.⁷¹ Colors absorbing at 570-578 $m\mu$ are given by skatole, *N*-acetyltryptamine,

⁶⁶ H. P. Rieder and M. Bohmer, *Experientia* **14**, 463-5 (1958).

⁶⁷ D. White and G. A. Vaughan, *Analyst* **82**, 597-9 (1957).

⁶⁸ L. A. Mokhov and N. S. Mareeva, *Lab. Delo* **8**, 29-31 (1962).

⁶⁹ Marjorie Knowlton, F. Curtis Dohan and Herbert Sprince, *Anal. Chem.* **33**, 666-8 (1960); J. M. Turner, *Biochem. J.* **78**, 790-2 (1961).

⁷⁰ T. A. Scott, *Biochem. J.* **80**, 462-4 (1961); E. McEvoy-Bowe, *Analyst* **88**, 893-4 (1963).

⁷¹ P. Byrom and J. H. Turnbull, *Talanta* **10**, 1217-21 (1963).

N-acetyltryptophan, and 2-(indol-3-yl)propionic acid, but they develop more slowly and are less intense than that due to indole.

p-Dimethylaminobenzaldehyde in aqueous trifluoroacetic acid reacts with indole and 3-substituted indoles. The colors are more stable than with the conventional Ehrlich reaction. Indole and skatole as impurities in methylnaphthalene are read additively in the infrared at 2.9 μ .⁷²

Procedure—*By nitrous acid. Air.* Pass 20 liters of air at the rate of 0.5 liter per minute through two absorption vessels, each containing 5 ml. of 50% ethanol. Treat the combined solutions, containing about 0.1 mg. of indole per ml., with 0.2 ml. of concentrated nitric acid and 0.2 ml. of 50% sodium nitrite solution. After 10 minutes, read at 525 $m\mu$. To remove interfering homologs, fractionate the sample in a column of an efficiency greater than 30 theoretical plates.

By p-dimethylaminobenzaldehyde. Mix a 2-ml. sample containing about 0.02 mg. of indole with 2 ml. of a 1% solution of *p*-dimethylaminobenzaldehyde in 10% trifluoroacetic acid solution. Store at 25° for 100 minutes and read at 563 $m\mu$.

Alternatively, dissolve 1.6 gram of *p*-dimethylaminobenzaldehyde in 125 ml. of 95% ethanol and 32 ml. of concentrated hydrochloric acid. Extract a 20-ml. sample with 40 ml. of 40-60° petroleum ether. Discard the aqueous layer. Add 10 ml. of the benzaldehyde reagent, shake vigorously, and read the aqueous layer at 560 $m\mu$.

By p-dimethylaminocinnamaldehyde. Extract a 10-ml. aqueous sample with 5 ml. of toluene. Mix a 4-ml. aliquot of the extract with 1 ml. of 0.5% dimethylaminocinnamaldehyde solution in propanol. Digest at 37° for 10 minutes. Add 0.5 ml. of 8:92 sulfuric acid-ethanol. After 10 minutes, add 4 ml. of 50% acetic acid. Cool, and shake thoroughly. Separate the aqueous acid layer and read at 625 $m\mu$ against a reagent blank.

By ferric chloride. Mix a 0.5-ml. sample solution containing a maximum of 0.012 mg. of indole with 0.1 ml. of a 0.3% sodium glyoxylate solution containing 0.01% of ferric chloride. Slowly add 2 ml. of sulfuric acid with shaking and heat for 3 minutes at 100°. Cool to room temperature and read in the range from 330-650 $m\mu$.

⁷² Seishi Tanaka, *Japan Analyst* **4**, 319-20 (1955).

INDOLE-3-ACETIC ACID

After extraction with chloroform and transfer to a buffer solution, indole-3-acetic acid is developed with persulfate and thioglycollic acid.⁷³

The presence of a large excess of antimonyl ion forms with indol-3-acetic acid a violet in hydrochloric acid, a purple in acetic acid.⁷⁴ The limit of determination is 1:100,000. There is no interference by tryptophan. But in hydrochloric acid flavones, anthocyanins, chlorophyll derivatives, and water-soluble carotenoids must be absent. Indole-3-acetic acid is read directly in the range of 230-300 m μ .⁷⁵

Procedure—*Urine. Free indole-3-acetic acid.* Mix 4 ml. of sample, 0.36 ml. of concentrated hydrochloric acid, and 10 ml. of chloroform. After shaking for 5 minutes, centrifuge for 10 minutes. As a buffer for pH 7, mix 6.06 ml. of 7.1% solution of anhydrous disodium phosphate with 3.94 ml. of 6.8% solution of monopotassium phosphate. Add 0.6 ml. of this to 9 ml. of the chloroform layer. Shake and centrifuge. Mix 0.4 ml. of the buffer extract with 2 ml. of acetic acid. Add 1 ml. of concentrated sulfuric acid and mix. Add 1 drop of saturated solution of potassium persulfate and 1 drop of 5% mercaptoacetic acid in acetic acid. After 10-15 minutes, read at 540 m μ .

Total indole-3-acetic acid. Mix the 4 ml. of sample with 0.36 ml. of concentrated hydrochloric acid and heat at 100° for 15 minutes to hydrolyze the conjugated form. Cool, add 10 ml. of chloroform, and proceed from "After shaking for 5 minutes . . ."

5-HYDROXYINDOLE-3-ACETIC ACID

An acidic extraction of the sample with diethyl ether removes 5-hydroxy-indole-3-acetic acid. The interference of melatonin and *N*-acetylserotonin, which may also be extracted, is avoided by previously making alkaline and extracting with diethyl ether. The fluorescence of 5-hydroxy-indole-3-acetic acid is then read at 540-550 m μ .⁷⁶

Procedure—Follow the procedure for serotonin in the presence of other 5-hydroxy and 5-methoxy indoles, page 157. Start at the beginning

⁷³ J. Fischl and S. Rabiah, *Clin. Chem.* **10**, 281-90 (1964).

⁷⁴ I. Ihász, *Magyar Kém. Foly.* **69**, 521-3 (1963).

⁷⁵ R. A. Fletcher and S. Zalik, *Nature* **199**, 903-4 (1963).

⁷⁶ W. B. Quay, *Anal. Biochem.* **5**, 51-9 (1963).

and end with, "In another centrifuge tube, combine 8 ml. of heptane and 0.5 ml. of 1:110 hydrochloric acid containing 0.5% of ascorbic acid." To the tube containing the sample, add 0.25 ml. of pH 10 buffer saturated with sodium chloride and diethyl ether. Immediately shake for 1 minute, centrifuge for 1 minute, and discard the ether phase. Add another 3 ml. of ether and repeat the shaking and centrifuging. Discard the ether phase. Add 0.15 ml. of 1:110 hydrochloric acid, mix, and add 3 ml. of diethyl ether. Immediately shake for 1 minute and centrifuge for 1 minute. Transfer the ether phase to the tube containing the heptane. Shake for 2 minutes and centrifuge. Read the fluorescence of the aqueous phase at 540-550 $m\mu$ with activation at 295 $m\mu$.

5-METHOXYINDOLE-3-ACETIC ACID

Acidic extraction with *p*-cymene recovers 5-methoxyindole-3-acetic acid. The accompanying melatonin is first removed from alkaline solution by a diethyl ether wash. The fluorescence is read at 540-550 $m\mu$.⁷⁷

Procedure—Follow the procedure for 5-hydroxyindole-3-acetic acid, ending with "Add another 3 ml. of ether and repeat the shaking and centrifuging. Discard the ether phase." Add 0.2 ml. of 1:110 hydrochloric acid. Mix, and add 3 ml. of *p*-cymene. Shake for 1 minute and centrifuge for 1 minute. Transfer the cymene phase to the tube containing heptane and shake for 2 minutes. Centrifuge, and read the fluorescence of the aqueous phase at 540-550 $m\mu$ with activation at 295 $m\mu$.

2-PHENYLINDOLE

A violet compound is formed when 2-phenylindole reacts with *p*-dimethylaminobenzaldehyde in acid alcohol solution.⁷⁸ The color is stable for 1 hour and decreases at the rate of 1% per hour after 2 hours. Phenols, aliphatic amines, and other aromatic amines give a color with the reagent.

Procedure—To prepare the reagent, dissolve 2.5 grams of *p*-dimethylaminobenzaldehyde in 1 liter of methanol containing 25 ml. of 1:2 hydrochloric acid. To 5 ml. of a methanol solution containing 10 mg. to 1 gram

⁷⁷ Loc. cit.

⁷⁸ M. V. Gilho-Tos, S. A. Previtera and E. M. Goodman, *Anal. Chem.* 36, 425-6 (1964).

of 2-phenylindole per liter, add 1 ml. of reagent. Shake, and read at 560 $m\mu$ after 50 minutes.

3,3-Bis(HYDROXYPHENYL)OXINDOLE

Potassium ferricyanide produces a color with 3,3-bis (hydroxyphenyl)-oxindole in the presence of dioxane.⁷⁹ An increase in dioxane content from 0.2 to 0.5 ml. increases the color intensity approximately 30%. Although the intensity of the color is reproducible, Beer's law is not followed.

Procedure—As a reagent, dissolve 2 grams of potassium ferricyanide and 2 grams of potassium carbonate in water and dilute to 1 liter. Dilute a dioxane solution containing 0.125-1 mg. of 3,3-bis(hydroxyphenyl)oxindole per 5 ml. of dioxane to 50 ml. with the reagent. Read with a green filter.

PYRIDINE

Upon addition of pyridine, the yellow dioxane solution of 2,2',4,4',6,6'-hexanitrodiphenylamine turns orange or red through dissociation of the acid.⁸⁰ The color, which appears immediately, is stable for at least 6 hours. The identification limit is 0.004 mg. per ml. The concentration limit is 0.01 mg. per ml. or 10 ppm. The dilution limit is 10^5 . The calibration curve prepared from samples containing 1-10 mg. of pyridine follows Beer's law.

Benzene, carbon tetrachloride, chloroform, and ethyl ether do not interfere. Ethanol, methanol, and acetone do not interfere if present below 20%. Over 10% concentration of water interferes, and if the pyridine concentration is small, over 5% concentration of water interferes. Inorganic and organic bases interfere. The reagent should be freshly prepared, since the hexanitrodiphenylamine turns orange in dioxane in 24 hours.

Cyanogen bromide is a common reagent for pyridine⁸¹ (Vol. IV, p. 243). It opens up the pyridine ring to give glutaeonic dialdehyde. This then condenses with barbituric acid to give a colored derivative.⁸²

⁷⁹ M. Kahane and O. Sackur, *Ann. pharm. franc.* **11**, 103-9 (1953).

⁸⁰ S. Kertes and V. Kertes, *Anal. Chim. Acta* **15**, 73-6 (1956).

⁸¹ T. Pavolini and G. Francesconi, *Chim. e Ind.* **40**, 544-7 (1958); B. Blanka, *Collection Czech. Chem. Commun.* **26**, 2442-5 (1961); H. Houben, *Brennst. Chemie* **44**, 299-301 (1963).

⁸² E. Asmus and H. F. Kurandt, *Z. anal. Chem.* **149**, 3-15 (1956); Walter Nielsch and Lieselotte Gießer, *ibid.* **171**, 401-10 (1960); Cf. G. V. Semukhina, V. D. Barskii, and V. V. Noskov, *Zh. analit. Khim.* **19**, 1155-8 (1964).

Pyridine and nicotine are determined simultaneously by conversion to glutacondialdehyde and condensation with barbituric acid.⁸³ Pyridine may be read in the ultraviolet in the presence of α -picoline, 2,3-lutidine, and quinoline after extraction with iso-octane. Samples that are fractions recovered from a cation-exchange column by eluting with hydrochloric acid in methanol are distilled to remove methanol.⁸⁴

Procedure—*By 2,2',4,4',6,6'-hexanitrodiphenylamine.* Samples containing 1-10 mg. of pyridine. Dissolve 0.22 gram of 2,2',4,4',6,6'-hexanitrodiphenylamine in 50 ml. of dioxane. To 8 ml. of the reagent, add a 2-ml. sample containing 1-10 mg. of pyridine. Read at 535 $m\mu$.

Samples containing 0.1-1 mg. of pyridine. Dissolve 2.2 grams of 2,2',4,4',6,6'-hexanitrodiphenylamine in 50 ml. of dioxane. To 8 ml. of the solution, add a 2-ml. dioxane solution of the sample containing 0.1-1 mg. of pyridine. Read at 540 $m\mu$.

By cyanogen bromide. Drugs. Mix the following: 1.25 ml. of buffer for pH 5, 0.5 ml. of 10% potassium cyanide solution, 2.5 ml. of 10% chloramine-T solution adjusted to pH 5, 6.25 ml. of 2% barbituric acid solution adjusted to pH 5, and 2.5 ml. of acetone. Add a sample containing up to 0.01 mg. of pyridine and dilute to 25 ml. with water. Read at 578 $m\mu$ for pyridine, 600 $m\mu$ for isonicotinic acid or 4-picoline, 600-615 $m\mu$ for isonicotinoylglycine, or 615 $m\mu$ for isonicotiniamide.

Coking gas. Shake a 1-liter sample with 25 ml. of 1:17 sulfuric acid for 5 minutes. Dilute the extract and water-washings to 100 ml. Take an aliquot equivalent to 0.001-0.025 mg. of pyridine and neutralize with 8% sodium hydroxide solution.

As a buffer for pH 7.5, mix 150 ml. of a 6.8% solution of monopotassium phosphate with 850 ml. of a 7.1% solution of anhydrous disodium phosphate. As sodium sulfanilate reagent, dissolve 2 grams of sulfanilic acid and 4 grams of sodium hydroxide in 500 ml. of water. Make alkaline to phenolphthalein with 16% sodium hydroxide solution. As cyanogen bromide reagent, mix 25 ml. of concentrated sulfuric acid and 80 ml. of water. Separately dissolve 23 grams of potassium bromate and 38 grams of potassium cyanide in 900 ml. of water. Add the diluted sulfuric acid. If the solution becomes colored on standing for 30 minutes, decolorize by additions of 10% potassium cyanide solution.

⁸³ E. Asmus and D. Papenfuss, *Z. anal. Chem.* **185**, 201-11 (1962).

⁸⁴ Ta-Chuang Lo Chang and Clarence Karr, Jr., *Anal. Chem.* **29**, 1617-9 (1957).

Add 5 ml. of the buffer, 5 ml. of sodium sulfanilate solution, and 3.5 ml. of cyanogen bromide solution. Shake for 1 minute and store at 22-28° in the dark for 60-80 minutes. Read at 465 $m\mu$ against a reagent blank.

Pyridine and nicotine simultaneously. To a 1-ml. aqueous sample containing less than 0.1 mg. of pyridine and less than 0.5 mg. of nicotine, add 1 ml. of 1% potassium cyanide solution and 5 ml. of 1% chloramine-T solution. Heat at 100° for 5 minutes and add 10 ml. of 1% aqueous barbituric acid and 25 ml. of phosphate buffer for pH 6.5. Dilute to 50 ml. and heat at 100° for 1 hour. Read pyridine at 578 $m\mu$ and nicotine at 509 $m\mu$. The latter requires a correction for the pyridine content.

PYRIDINE AND QUINOLINES

After extraction from coal tar with sulfuric acid, the solution containing total pyridine and quinolines is made alkaline and the free bases are extracted from the alkaline solution into isooctane. The concentrations of total quinolines and pyridines are determined by the absorbances at 316.5 $m\mu$ and 260 $m\mu$.⁸⁵ When the bases are extracted from the distillate, a small amount of acid-soluble phenols and hydrocarbons is carried over. They absorb at 250-260 $m\mu$ and may interfere. To eliminate this error, wash the acid extract with isooctane and hold all phenols in the alkaline solution during the final extraction of the bases with isooctane. The spectrum from tar bases using this method shows an additional peak at 252 $m\mu$, the result of a small amount of aniline derivatives. When the pyridine base is a chemical individual, apply Table 29.

Quinoline and isoquinoline are read in 0.2% sodium hydroxide solution at less than 0.025 mg. of each per ml.⁸⁶ The first is read at 289 and 312.7 $m\mu$, and the iso compound, at 319 $m\mu$. The presence of sodium sulfate and sodium carbonate does not affect either one. Quinoline and tetrahydroquinoline are read in carbon tetrachloride at 10.66 μ and 9.15 μ , respectively.⁸⁷

Procedure—Tar distillates. For samples containing 1-2% of base by weight, extract a 10-ml. sample with one 20-ml. portion of 10% sulfuric acid and two 10-ml. portions of the acid. Combine the acid extracts. Ex-

⁸⁵ Ta-Chuang Lo Chang and Clarence Karr, Jr., *Anal. Chem.* **30**, 971-2 (1958).

⁸⁶ Motohisa Furusawa, Tsuguo Takeuchi, and K. Kamijo, *J. Tar Ind. Assoc. Japan* **11**, 238-41 (1959); *Kogyo Kagaku Zasshi* **63**, 616-19 (1960).

⁸⁷ H. Pobiner, *Appl. Spectroscopy* **17**, 79 (1963).

TABLE 29. ABSORPTIVITIES OF PYRIDINE BASES IN 10% SULFURIC ACID

<i>Pyridine Bases</i>	<i>A, At Wave Length</i>	
	<i>260 mμ</i>	<i>316.5 mμ</i>
Pyridine	17.44	0
2-Methylpyridine	25.43	0
3-Methylpyridine	23.47	0
4-Methylpyridine	14.22	0
2,3-Dimethylpyridine	26.86	0
2,4-Dimethylpyridine	22.41	0
2,6-Dimethylpyridine	26.74	0
3,5-Dimethylpyridine	22.87	0
2-Ethylpyridine	24.47	0
4-Ethylpyridine	13.07	0
5-Ethyl-2-methylpyridine	20.99	0
3-Ethyl-4-methylpyridine	18.87	0
2,4,6-Trimethylpyridine	20.33	0
Av.	21.25	0
<i>Quinoline Bases</i>		
Quinoline	24.23	9.84
2-Methylquinoline	23.90	24.71
4-Methylquinoline	27.69	8.97
6-Methylquinoline	17.79	12.39
7-Methylquinoline	19.16	18.95
8-Methylquinoline	12.56	12.87
2,4-Dimethylquinoline	24.24	17.25
2,6-Dimethylquinoline	21.25	13.56
Isoquinoline	28.67	27.27
3-Methyl-isoquinoline	27.08	13.69
Av.	23.24	15.95

tract the oil residue with 10 ml. of 10% sodium hydroxide solution to remove tar acids, which interfere with complete extraction of the tar bases. Discard the alkaline layer and extract the oil layer with one 20-ml. portion of 10% sulfuric acid and two 10-ml. portions of acid. Add these extracts to the original combined acid extracts. Dilute the total extracts to 100 ml. with 10% sulfuric acid.

Wash 25 ml. of the acid extract of the sample three times with 25 ml. portions of iso-octane to remove any residual neutral oil. Adjust the acid solution to 25 ml. with water, if necessary. Pipet 2 ml. of the acid solution into a beaker containing a few ml. of water and place the beaker in ice water. Add potassium hydroxide pellets with constant stirring until

the pH reaches 12. Dilute to 25 ml. with water. Extract a 5-ml. aliquot with three 10-ml. portions of isooctane. Combine the extracts and read at 260 and 316.5 m μ .

Calculate as follows:

$$Q = \frac{A_{316.5}}{15.95} \times F_1 \times F_2$$

$$P = \frac{A_{260} - [(A_{316.5}/15.95) \times 23.24]}{21.25} \times F_1 \times F_2$$

in which Q is the total quinolines in grams; P is the total pyridines in grams; $A_{316.5}$ is the absorbance at 316.5 m μ ; A_{260} is the absorbance at 260 m μ ; F_1 is the dilution factor for final isooctane extract, which is 1, using the above procedure; and F_2 is the dilution factor for sample, which, in the above procedure, is 7.5 for distillate and 3.75 for crude tar base.

Crude Tar Base. Dissolve a 0.1 gram sample in 1:9 sulfuric acid and dilute to 50 ml. with 1:9 sulfuric acid. Follow the procedure for tar distillates, starting with "Wash 25 ml. of the acid extract. . . ."

PICOLINES AND 2,6-LUTIDINE, 2,6-DIMETHYLPYRIDINE

The solutions of α , β , and γ picoline and 2,6-lutidine in 10% sulfuric acid are read in the ultraviolet at 260 m μ . For the absorptivities, see Table 29. There are maxima for β -picoline at 260 m μ , γ -picoline at 252.5 m μ , and 2,6-lutidine at 270 m μ .⁸⁸ A system of simultaneous equations is solved for any component present to more than 4.5%. They are also determinable by cyanogen bromide and barbituric acid by a method given for pyridine, page 463.

For infrared reading, the transmissions given below are preferable to the maxima cited.⁸⁹

	Maximum μ	Recommended μ
α -Picoline	13.39	13.73
β -Picoline	12.81	12.86
γ -Picoline	12.62	12.52

⁸⁸ M. M. Bragilevskaya, I. E. Kogan, and M. E. Nelmark, *Koksohim.* 1962, (4), 44-7.

⁸⁹ Gianfranco Fabbri, *Ann. Chim. (Rome)* 52, 983-4 (1962).

β -PICOLINE, β -METHYLPYRIDINE

β -Picoline is developed with a bromide-thiocyanate reagent in the presence of ammonium sulfanilate.⁹⁰

Procedure—As reagent, discharge most of the color of saturated bromine water with a 10% solution of ammonium thiocyanate and complete with a 1% solution. Add calcium carbonate until a slight cloudiness persists, and use within 24 hours.

Grind 0.5 gram of fresh tissue with 10 ml. of 1:110 hydrochloric acid. Add 15 ml. of 0.4% sodium hydroxide solution and steam distil. Collect 20 ml. in 2 ml. of 1:110 hydrochloric acid. Nearly neutralize a 6-ml. aliquot to phenolphthalein with 4% sodium hydroxide solution, finishing off with a 0.4% solution. Dilute to 7 ml. with water. Add 0.2 ml. of 1:88 acetic acid.

As a reagent, mix 2 ml. of 1:4 ammonium hydroxide with 0.1 gram of sulfanilic acid and dilute to 150 ml. with water. Add 2.5 ml. of this reagent, and if a pink color appears, neutralize. Add 2.5 ml. of the thiocyanate-bromide reagent and read after 5 minutes.

ACRYLONITRILE-METHYLVINYLPYRIDINE COPOLYMERS

These polymers are hydrolyzed and the two separate ingredients analyzed. Nitrile is determined by Kjeldahl-type distillation followed by titration as ammonia. Pyridine is determined by partial hydrolysis of the test substance to solubilize, solution in sulfuric acid, and reading in the ultraviolet.⁹¹ Naturally, it is applicable with substituents other than methyl.

Procedure—Mix 0.1 gram of sample with 50 ml. of 54% sulfuric acid. Reflux for 45 minutes to partially hydrolyze. Cool, and dilute to such a volume with concentrated sulfuric acid that the sulfuric acid is 90% and the methyvinylpyridine 1-2 mg. per 100 ml. The water content may be adjusted as necessary to accomplish this. Read the absorbance over the range of 230-300 m μ . Draw a tangent to the absorbance at 295 and 235 m μ . The corrected absorbance is the distance from this line to the peak at

⁹⁰ A. G. Khalmurodov, *Lab. Delo* **10**, 224-5 (1964).

⁹¹ Camille Stafford Jr. and Paul E. Toren, *Anal. Chem.* **31**, 1687-9 (1959).

270 $m\mu$. For a nonrecording instrument, read at 235, 270, and 295 $m\mu$, and proceed as above.

$$\text{Corrected absorbance} = A_{270} - A_{295} - 0.417 (A_{235} - A_{295})$$

NIACIN, PYRIDINE-3-CARBOXYLIC ACID, NICOTINIC ACID

Cyanogen bromide with various additions has been extensively used as a reagent for niacin⁹² (Vol. IV, pp. 247-259). When treated with cyanogen bromide, the maximum is at 370 $m\mu$, while nicotinamide has a low value at that wave length but a maximum at 370 $m\mu$.⁹³ After treatment with cyanogen bromide, the product may be coupled with *p*-aminobenzoic acid. Only nicotine and pyridine interfere. In the case of urine, preliminary oxidation with permanganate cuts down background interference.⁹⁴

In the presence of potassium cyanide, nicotinic acid gives a yellow condensation product with chloramine-T.⁹⁵ The concentration limits of the method are 0.001-0.01 mg. per ml., and Beer's law is followed. The extinction is influenced by the time of reaction.

Nicotinic acid and isonicotinic acids are separately determined spectrophotometrically.⁹⁶ As the ethyl esters, the levels for reading are 239 and 285 $m\mu$. Correspondingly, the butyl esters absorb at 230 and 285 $m\mu$. This method permits their separate estimation.

The color with sodium benzene sulfonate chloramide may be used to determine nicotinic acid.⁹⁷ Liberation of combined niacin by hydrolysis may be attained under alkaline or acid conditions. The latter has been indicated as being more accurate.⁹⁸ Niacin acetate is read at pH 5 and 262 $m\mu$.⁹⁹ Vitamins A, E, B₁, B₆, and C do not interfere.

Procedure—*By cyanogen bromide. Blood.* To prepare the acid precipitant, dissolve 13 grams of cadmium sulfate and 63 ml. of concentrated hydrochloric acid in water and dilute to 1 liter. As a reagent, dissolve 10.7 grams of potassium bromide and 4 ml. of bromine in water, and dilute

⁹² A. M. Petrun'kina, *Lab. Delo* **9**, 1-4 (1963).

⁹³ G. Quagliariello and G. Porcellati, *Boll. soc. ital. biol. sper.* **30**, 64-8 (1954).

⁹⁴ D. K. Chaudhuri, *Indian J. Med. Research* **39**, 491-505 (1951); *Science and Culture* **17**, 270-1 (1951).

⁹⁵ J. R. Peknice, *Ceskoslov. farm.* **2**, 354-7 (1953); cf. Oden Hever, *Z. Physiol. Chem.* **325**, 275-6 (1961).

⁹⁶ Nobuo Sohma, *Ann. Rept. Takamini Lab.* **7**, 97-102 (1955).

⁹⁷ Oden Hever, *Z. Physiol. Chem.* **325**, 275-6 (1961).

⁹⁸ B. Gassmann and J. Janicki, *Ernahrungsforschung* **8**, 377-86 (1963).

⁹⁹ L. Campaioli and M. Giannini, *Farmaco (Pavia) Ed. prat.* **11**, 229-34 (1956).

to 100 ml. Treat the reagent with potassium thiocyanate solution until the color disappears. Add 1 drop of phenolphthalein and neutralize with sodium hydroxide. Into each of four 3-ml. tubes, place 0.3 ml. of 4.4% sodium hydroxide solution, 0.5 ml. of water, and 0.3 ml. of blood. Mix. To 2 tubes, add 0.3 ml. of a solution of nicotinic acid containing 0.1 mg. per ml. To all 4 tubes, add 0.5 ml. of acid precipitant. If the solution is not chocolate color, or if a precipitate does not form, test 1 drop on filter paper treated with 0.1% bromothymol blue solution in ethanol to determine if the correct green-yellow color appears. If necessary, adjust the pH with acid precipitant or 4.4% sodium hydroxide solution. Dilute each solution to 3 ml. and centrifuge for 10 minutes. To the supernatant liquid, add 0.3 ml. of 40% sodium hydroxide solution. The final mixture should be *N* with respect to sodium hydroxide.

Heat at 100° for 45 minutes to hydrolyze combined nicotinic acid, and carefully neutralize with 2:3 and then 1:5 hydrochloric acid. To each solution, add 0.5 gram of monobasic potassium phosphate and let the tubes stand in warm water to dissolve the phosphate. Dilute with water to twice the original volume after protein precipitation. Into two additional tubes, add 0.5 gram of monobasic potassium phosphate and 6 ml. of water to serve as blanks to determine the extinction of cyanogen bromide solution. Centrifuge all 6 tubes for 10 minutes. Transfer a 2-ml. portion of the supernatant liquid from each tube into each of 2 tubes. In one of each pair, place 1 ml. of water, and if there is a trace of cloudiness, centrifuge for 10 minutes. Place the second of each pair in a bath at 100° and add 1 ml. of cyanogen bromide reagent to each. Heat at 100° for 5 minutes, cool in ice water, and centrifuge for 10 minutes. Read each solution containing cyanogen bromide at 351.5 m μ , using the other tube of each pair to set the instrument to zero.

*Urine.*¹⁰⁰ Digest a 20-ml. sample with 4 ml. of concentrated hydrochloric acid for 45 minutes at 100°. Cool to room temperature and centrifuge. Dilute the supernatant liquid to 25 ml. and extract with 12.5 ml. of water-saturated isobutanol by shaking for 2-3 minutes. Allow the mixture to settle. Draw off the water layer and heat it at 100° to remove the last traces of isobutanol. Add 4% potassium permanganate solution, 3-4 drops at a time, until there is no color change. Cool the extract to room temperature and adjust the pH to 6.6-6.8. Dilute the 65 ml. of extract with 35 ml. of 95% ethanol. Place in a refrigerator for 30 minutes and centrifuge.

¹⁰⁰ D. K. Chaudhuri, *Ann. Biochem. and Exptl. Med.* 12, 119-34 (1952).

Adjust the pH of a 10-ml. portion to 6.6-6.8. Add 2 ml. of freshly prepared cyanogen bromide solution. Add 2 ml. of 5% solution of *p*-amino-benzoic acid and 0.3 ml. of 1:3 hydrochloric acid. Read after 1-2 minutes.

By chloramine-T. Animal organs. Grind the sample and extract with three times its weight of 8% sodium hydroxide solution at 100° for 30 minutes. Adjust the pH to 6-6.2 with hydrochloric acid and centrifuge. Combine a 1-ml. portion of the sample equivalent to approximately 250 mg. of sample with 9 ml. of acetone or 95% ethanol. To 4 ml. of this, add 3 ml. of water and evaporate the solvent *in vacuo*. Dilute the residue to 25 ml. with 2% monobasic potassium phosphate solution. To a 5-ml. aliquot, add 2.7 ml. of 15% chloramine-T solution and 0.6 ml. of 5% potassium cyanide solution. Dilute to 10 ml. with water and let stand for 5 minutes in ice water, then for 3 minutes in water at 20°. Read at 440 m μ within 2 minutes, avoiding exposure to light.

Ointment containing benzyl nicotinate. Heat for 2 hours at 100° with 2.8% potassium hydroxide solution to saponify. Acidify. Defat by ether extraction.¹⁰¹ Complete as for urine, from "Adjust the pH of a 10-ml portion . . ."

4-PYRIDOXIC ACID, 2-METHYL-3-HYDROXY- 4-CARBOXY-5-HYDROXYMETHYLPYRIDINE

The estimation of 4-pyridoxic acid consists of four steps: (1) delactonization to eliminate the problem of spontaneous delactonization at room temperature in the hydrochloric acid used to eluate the 4-pyridoxic acid; (2) lactonization at 100° with hydrochloric acid; (3) adjustment of the pH to 9 for optimum reading of fluorescence; and (4) dilution and reading of the fluorescence.¹⁰² Ion-exchange chromatography removes from 40-75% of the fluorescence of the sample with no effect on the 4-pyridoxic acid. Chlorotetracycline interferes with the determination. Otherwise, there is no interference by metabolites with each other or by penicillin sulfate, streptomycin sulfate, chloramphenicol, or bilirubin.¹⁰³ Vanillin does not interfere. Beer's law is followed for 0.000067-0.000201 mg. of 4-pyridoxic acid.

¹⁰¹ I. Bors, *Acad. Rep. Populare Romane, Filiala Lasi, Studii Cercetari Stiint Chim.* 13, 277-8 (1962).

¹⁰² Saranya K. Reddy, May S. Reynolds and J. M. Price, *J. Biol. Chem.* 233, 691-6 (1958); M. J. Woodring, D. H. Fisher and C. A. Storvick, *Clin. Chem.* 10, 479-89 (1964).

¹⁰³ P. D. Starshov, *Lab. Delo* 8, No. 6, 37-42 (1962).

A method of development of 4-pyridine derivatives with chloramine-T, potassium cyanide, and barbituric acid shown for niacin is applicable to isonicotinic acid.¹⁰⁴ Reading is at 600 m μ .

Procedure—Urine. Mix Dowex 1, 10% cross linkage, 200-400 mesh, chloride with water and allow to sediment. Remove the very fine and very coarse particles by decantation. Convert to the hydroxide form by washing 750 ml. of the packed resin in a column with 24 liters of 8% sodium hydroxide solution with a flow rate of 500 ml. per hour. Wash the column with 5 liters of water. Prepare the resin fresh weekly, as the hydroxide form darkens during storage.

Allow Dowex 50, 12% cross linkage, 200-400 mesh to sediment in the same manner. Wash 1500 ml. of the packed resin in a column with 25 liters of 2:1 hydrochloric acid, 12 liters of 1:2 hydrochloric acid, and 10 liters of water at a flow rate of 750 ml. per hour.¹⁰⁵

To assemble the chromatography columns, seal 1.2 \times 20-cm. glass tubes to the bottom of 125 ml. Erlenmeyer flasks. Place glass wool plugs above constrictions near the bottom of the tubes. Pipet a slurry of resin into each column to form a packed layer, 3 cm. long. Wash the Dowex 50 column successively with 50 ml. of 2:3 hydrochloric acid, 50 ml. of 1:5 hydrochloric acid, and 50 ml. of water. Wash the Dowex 1 column with 50 ml. of 1:5 hydrochloric acid and 50 ml. of water. Operate the columns without added pressure.

Collect 24-hour samples in amber bottles containing 25 ml. of toluene. Store an aliquot of the sample at 0° until use. Adjust about 4% of the sample to pH 10.6 with saturated sodium hydroxide solution and filter. To duplicate portions of the filtrate representing 1% of the total sample, add 0.025 mg. of synthetic 4-pyridoxic acid and 1.5 ml. of 5% ammonium hydroxide. Dilute to 40 ml., stir well, and add to the Dowex 1, chloride column. Rinse the tubes containing the sample with 20 ml. of water and use the water to wash the sample through the column. Elute the 4-pyridoxic acid with 50 ml. of 1:239 (0.05 *N*) hydrochloric acid and pass the effluent directly into the Dowex 50 column. Wash the sample through the Dowex 50 column with 20 ml. of water and elute the 4-pyridoxic acid with 50 ml. of 1:5 hydrochloric acid.

For delactonization, pipet 4 ml. of the 1:5 hydrochloric acid eluate from the Dowex 50 tube into duplicate centrifuge tubes. Add 2 ml. of 20% sodium hydroxide solution to each and heat for 5 minutes at 100°.

¹⁰⁴ Walter Nielsch, *Chemiker-Ztg.* **82**, 137-8, 329-41, 494-9 (1958).

¹⁰⁵ J. M. Price, *J. Biol. Chem.* **211**, 117-124 (1954).

Cool. Add 2 ml. of 1:10 hydrochloric acid to neutralize the base. To lactonize the sample, add 2 ml. of 2:3 hydrochloric acid to one tube only and heat for 15 minutes at 100°. Cool. The second tube serves as a blank. After lactonization, make the sample slightly alkaline by addition of 11 ml. of 4% sodium hydroxide solution, and dilute to 30 ml. with water. Dilute approximately 2 ml. of the solution immediately to 10 ml. with 1% sodium borate solution. The pH of the final solution is 9 ± 0.3 , which is optimal for fluorescence measurements.

Since considerable lactonization occurs in acid solution at room temperature, the blanks are prepared differently from the sample. To prepare the blank, add 11 ml. of 4% sodium hydroxide solution to the second tube of the pair prepared above. Add 2 ml. of 1:10 hydrochloric acid, 2 ml. of 2:3 hydrochloric acid, and dilute to 30 ml. with water. Immediately dilute a 2-ml. aliquot to 10 ml. with 1% sodium borate solution.

Read the fluorescence with a Coleman model 12 photofluorometer, using filters B-1 and PC-1. Using a spectrophotofluorometer, the optimum activating wave length is 350 m μ and the fluorescence peak is at 450 m μ .

QUINOLINIC ACID, PYRIDINE-2,3-DICARBOXYLIC ACID

A yellow ferrous chelate complex is formed by reaction of ferrous ion with the ring nitrogen and the α -carbonyl group of quinolinic acid.¹⁰⁶ Picolinic acid gives a similar reaction. Nicotinic acid, phthalic acid, and proline do not react. Reading at 420 m μ avoids interference from 3-hydroxyanthranilic acid and substances absorbing in the near ultraviolet. Beer's law is followed for 1-10 micromoles in 5 ml. of final volume.

Procedure—To 1 ml. of 10% trichloroacetic acid extract containing 1-10 micromoles of quinolinic acid, add 1 ml. of 3.9% ferrous ammonium sulfate solution in 1:920 sulfuric acid, 0.05 ml. of 2-mercaptoethanol, and 2 ml. of 9% sodium acetate solution. Heat for 15 minutes at 75°, cool to room temperature, and read at 420 m μ against a reagent blank.

IDOCHLORHYDROXYQUINOLINE

The reaction of iodochlorhydroxyquin with ferric iron at a pH of between 1-2 produces a colored complex read at 650 m μ .¹⁰⁷ This method

¹⁰⁶ M. Rabinovitz, R. A. Fineberg and D. M. Greenberg, *Arch. Biochem. and Biophys.* **42**, 197-203 (1953).

¹⁰⁷ Jack Cohen and Elmer Kluchesky, *J. Pharm. Sci.* **52**, 693-4 (1963).

is applicable to iodochlorhydroxyquin ointment in hydrocarbon or hydrophilic ointment bases.

Procedure—To prepare the iron reagent, add 1 gram of ferric chloride and 1 ml. of concentrated hydrochloric acid to water, and dilute to 1 liter with water. Add about 3 grams of the sample to a tared flask and determine the sample weight. Heat with 50 ml. of acetone at 100°, with occasional swirling, until the sample melts. Stopper, shake vigorously, and let cool to room temperature. Dilute to 100 ml. with acetone and mix well. Filter a 3-ml. aliquot into a 25-ml. flask, using a pipet covered with a cotton pledget. Evaporate the acetone at 100° and chill the residue in an ice bath until it solidifies. To the residue, add 20 ml. of methyl Cellosolve, and swirl to disperse the solid. Do not shake vigorously. Add 2 ml. of the ferric iron reagent, dilute to 25 ml. with methyl Cellosolve, and mix well. Prepare a reagent blank with 2 ml. of ferric iron reagent diluted to 25 ml. with methyl Cellosolve. Remove most of the oil globules by inserting a cotton swab on the end of a glass rod into the solution in the neck of the flask. Read the clear solution at 650 $m\mu$ against the reagent blank.

METHAQUALONE, 2-METHYL-3-(*o*-TOLYL)QUINAZOLONE

After hydrolysis of the test substance the product is coupled with diazotized sulfanilic acid for reading.¹⁰⁸ Beer's law is applicable up to 0.1 mg. per ml.

Procedure—Add sufficient hydrochloric acid to make the sample 1:10 in that acid. Hydrolyze at 100° for an hour. Cool, and make weakly alkaline with 4% sodium hydroxide solution. Dilute with Clark and Lubs buffer for pH 5.0 (Vol. I, p. 174). As reagent, diazotize 1 ml. of 1% sulfanilic acid in 1:10 hydrochloric acid with 0.25 ml. of 1% sodium nitrite solution and keep in ice water for 2 minutes. Mix 5 ml. of buffered sample with this diazobenzenesulfonic acid reagent and warm at 70° for 30 minutes. Read at 400 $m\mu$.

PYRIDOXAL, 2-METHYL-3-HYDROXY-4-ALDEHYDE-5-HYDROXYMETHYLPYRIDINE

Aldehyde and phenol condense to form a chromogenic complex in the presence of a condensing agent such as concentrated hydrochloric or sul-

¹⁰⁸ Masaru Nakano, *Yakuzaigaku*, 22, 267-9 (1963).

furic acid. Only pyridoxal of the compounds related to vitamin B₆ possesses a phenolic hydroxyl as well as an aldehyde group, making it possible by this reaction to distinguish pyridoxal from pyridoxine, pyridoxamine, and pyridoxic acid.¹⁰⁹ In concentrated sulfuric acid, an intense yellow color is formed with peaks at 335 and 390 m μ . Beer's law is followed for 0.01-0.1 mg. of pyridoxal at 390 m μ .

Pyridoxal is readily determined in strongly alkaline solutions in the presence of alanine, glutamic acid, pyruvic acid, α -ketoglutaric acid, and other similar amino and keto acids as well as pyridoxine and pyridoxamine.¹¹⁰

Procedure—*In strong acid.* Dissolve a sample expected to contain 0.1 mg. of pyridoxal hydrochloride in 1 ml. of water and add 4 ml. of concentrated sulfuric acid. Read at 390 m μ .

In strong alkali. To a 1-ml. sample containing 0.02-0.4 mg. of pyridoxal hydrochloride, add 0.6 ml. of acetone and 0.4 ml. of 40% sodium hydroxide solution. Mix, and read at 420 m μ after 15 minutes against a reagent blank. The absorption conforms to Beer's law over this range.

NICOTINAMIDE, NIACINAMIDE, PYRIDINE-3-CARBOXYLIC ACID AMIDE

Nicotinamide is read fluorescently after treatment with cyanogen bromide.¹¹¹ Thiamine must be removed by fuller's earth and surfactants extracted with chloroform. *N'*-Methylnicotinamide must be separated by chromatography prior to treatment with cyanogen bromide.¹¹² Then treatment at pH 9-9.5 gives a maximum at 398 m μ .¹¹³ Nicotinic acid interferes if it is three times the niacinamide content.¹¹⁴ Pyridine and some of its α - and β -substituted derivatives, and *N*-methylnicotinamide, give interfering colors. Nicotinamide is read in about 5% solution along

¹⁰⁹ Victor E. Levine and Robert N. Sass, *Anal. Chim. Acta* **20**, 137-46 (1959).

¹¹⁰ Frederick P. Siegel and Martin I. Blake, *Anal. Chem.* **34**, 397-8 (1962).

¹¹¹ Keiji Inami and Akihide Ohara, *Vitamins* (Kyoto) **12**, 378-87 (1957).

¹¹² Yahito Kotake, Minoru Tsuji, and Norihiko Nasegawa, *J. Vitaminol* (Kyoto) **6**, 271-7 (1960).

¹¹³ Isamu Uchiyumi and Masayoshi Samejima, *Tanabe Seiyaku Kenkyu Nempo* **2**, No. 2, 42-6 (1957).

¹¹⁴ O. Pelletier and J. A. Campbell, *J. Pharm. Sci.* **50**, 926-8 (1961); *ibid.* **51**, 594-5 (1962).

with *N,N*-diethylnicotinamide, nikethamide, by adding copper sulfate solution.¹¹⁵

A purple color forms when niacinamide is treated with cyanogen bromide and barbituric acid in a potassium dihydrogen phosphate buffer (cf. Vol. IV, p. 257). Potassium dihydrogen phosphate is a suitable extractant because it does not cause hydrolysis of the niacinamide. Beer's law is followed for up to 0.01-0.0125 mg. of niacinamide per ml. The most suitable concentration for the procedure is 0.005-0.01 mg. per ml.

Niacin does not interfere unless present at three times the concentration of the amide. α -Substituted pyridine derivatives do not react. Nicotinic acid ethyl ester and the dimethyl amide of nicotinic acid give the same purple color as niacinamide with the reagents. *N'*-methyl nicotinamide produces a purple color only at high concentrations. None of these interfering substances are commonly found in multivitamin preparations. The purple color developed with 1,2-naphthoquinone sulfonate in acetone is suitable for direct reading.¹¹⁶

For reading nicotinamide in the ultraviolet in the presence of thiamine and pyridoxine, see page 518. After steam distillation, saponification, and paper chromatography, nicotinamide has been determined in condensed tobacco smoke.¹¹⁷

Procedure—By fluorescence. Mix 2 ml. of a solution of nicotinamide containing 10 mg. of phosphate buffer for pH 7 per ml. with 1 ml. of 10% cyanogen bromide reagent. Heat at 80° for 7 minutes. Cool, and read at 287-397 m μ .

By cyanogen bromide. Multivitamin preparations. To prepare the buffered barbituric acid solution, add 100 ml. of 3% potassium dihydrogen phosphate solution to 2 grams of barbituric acid and let stand for 1 hour with occasional shaking. Filter before use.

Measure the sample, grinding if in tablet form. Add a volume of 0.3% potassium dihydrogen phosphate equal in ml. to at least twice the mg. of niacinamide. For capsule samples, add 2 ml. of ethylene dichloride to aid in dispersion. If the sample is not readily soluble, shake and heat.

¹¹⁵ Aluisio Marques Leal, Maria Julia M. da Silva and Jacqueline Costa Santos, *Congr. Luso-Españ. farm.* II Congr., 2, 1a Sec. 202-11 (1952).

¹¹⁶ J. P. S. Sami, R. B. Chakravarty and G. K. Ray, *Indian J. Pharm.* 26, 165-8 (1964).

¹¹⁷ R. Wahl, *Z. anal. Chem.* 204, 25-8 (1964).

Dilute to a concentration of approximately 0.005 mg. per ml. with 0.3% potassium dihydrogen phosphate solution, and filter if necessary.

To a 1-ml. aliquot containing about 0.005 mg. of niacinamide add 0.5 ml. of 10% cyanogen bromide solution. Mix, stopper, and let stand for 25-30 minutes. Add 10 ml. of barbituric acid buffer and mix by swirling. If the barbituric acid is not to be added immediately, store the tube in crushed ice to stabilize the cyanogen bromide reaction. Read at 550 $m\mu$ within 2-4 minutes after the addition of the barbituric acid.

By 1,2-naphthaquinone-4-sulfonate. To a sample containing about 5 mg. of nicotinamide, add 2.5 ml. of 1:3 hydrochloric acid and dilute to 10 ml. with water. Reflux for 1 hour, cool, and dilute to 10 ml. To a 3-ml. aliquot, add 2 ml. of water, 3 ml. of 10.6% sodium carbonate solution, and 1 ml. of 0.5% solution of the sodium salt of 1,2-naphthaquinone-4-sulfonate. Maintain at 80° for an hour. Cool, and add 1 ml. of acetone, 1.5 ml. of 1:3 hydrochloric acid, and 0.5 ml. of 2.5% sodium thiosulfate solution. After a purple color develops, dilute to 20 ml. with acetone and read at 540 $m\mu$ against a blank.

N'-METHYLNICOTINAMIDE, *N'*-METHYLNIAcinAMIDE

After separation from *N*-methyl-2-pyridone-5-carbonamide in urine samples, *N*-methylnicotinamide is determined by the orange diazo dye formed by coupling with *N*-1-naphthylethylenediamine.¹¹⁸

By condensation with acetone in alkaline solution, *N'*-methylniacinamide forms a naphthyridine derivative. This is read by fluorescence.¹¹⁹ There is no interference by metabolites or by chlortetracycline, penicillin sulfate, streptomycin sulfate, chloramphenicol, or bilirubin. After the condensation, the addition of small amounts of hydrogen peroxide increases the fluorescence. Larger amounts of peroxide cause a decrease. Felosan, *N*-methylnicotinamide, with the methyl on the amido nitrogen, is read fluorimetrically¹²⁰

N'-Methylnicotinamide in urine is read fluorimetrically after appropriate treatment.¹²¹ The reaction with cyanogen bromide and barbituric acid in a monopotassium phosphate buffer is applicable for

¹¹⁸ W. I. M. Holman, *Biochem. J.* **56**, 513-20 (1954); A. W. M. Indemans and H. E. J. Radernakers, *Pharm. Weekblad.* **95**, 377-93 (1960).

¹¹⁹ Harold L. Rosenthal, *Science* **120**, 231 (1954); P. D. Starshov, *Lab. Delo* **8**, No. 6, 37-42 (1962).

¹²⁰ Taiichi Asami, *J. Vitaminol* (Osaka) **3**, 189-202 (1957).

¹²¹ P. D. Starshov, *Lab. Delo* **8**, No. 6, 37-42 (1962).

reading at 550 $m\mu$,¹²² page 475. N'-methylnicotinamide is condensed with methylethyl ketone in cold 35% urea solution and read fluorescently.¹²³ It will detect one microgram. There is no interference by indole, pyridoxal, thiamine, folic acid, or trigonelline. Negligible fluorescence is produced by riboflavine.

Procedure—*By diazotizing and coupling. Urine.* To activate Decalso, stir with two 10-volume portions of 3% acetic acid for 10 minutes each. Treat for 15 minutes with 5 volumes of a neutral 25% potassium chloride solution. Repeat the acid washes with two additional 10-volume portions of 3% acetic acid for 10 minutes each. Wash with water, ethanol, and ether, and dry in air.¹²⁴ To prepare the phosphate buffer at pH 7, mix 40 ml. of anhydrous dibasic potassium phosphate containing 9.078 grams per liter and 60 ml. of monobasic sodium phosphate monohydrate containing 9.465 grams per liter.

Collect 24-hour specimens under toluene and, if not used immediately, preserve with 10 ml. of glacial acetic acid, and store in a refrigerator. Measure a suitable sample into two tubes. To tube 2, add a known amount of N-methylnicotinamide chloride, usually 0.05-0.1 mg. To each tube, add 15 ml. of buffer and dilute to 30 ml. with water. Pour the sample onto adsorption columns containing 2 grams of activated Decalso. Wash with three 5-ml. portions of water, drying *in vacuo* after each addition. Elute the adsorbed N-methylnicotinamide with hot 25% potassium chloride solution and collect 10 ml. of eluate. Develop 2-ml. samples of eluate from column 1 representing the rest, and 2-ml. samples from column 2 representing the recovery blank, by diazotization and coupling with N-1-naphthylethylene diamine. See N-methyl-2-pyridone-5-carboxamide, page 551. Use 6 ml. of sodium hydroxide in place of 4 ml. in the preparation of the sodium hypobromite reagent. Start at "Dilute the sample and the recovery tube . . ." Read after 10 minutes at 500 $m\mu$.

Fluorimetrically. Urine. Dilute a 1-ml. sample with 5 ml. of water. Add 0.5 ml. of acetone and 0.2 ml. of 24% sodium hydroxide solution. After standing for 5 minutes, add 0.3 ml. of 1:1 hydrochloric acid. Heat at 100° for 2 minutes. Cool, and add 1 ml. of 20% monopotassium phosphate solution and 7 ml. of water. Read fluorimetrically.

¹²² O. Pelletier and J. A. Campbell, *J. Pharm. Sci.* **50**, 926-8 (1961).

¹²³ O. Pelletier and J. A. Campbell, *Anal. Biochem.* **3**, 60-7 (1962).

¹²⁴ Melvin Hochberg, Daniel Melnick and Bernard L. Oser, *J. Biol. Chem.* **158**, 265-77 (1945).

Felosan. Dilute a urine sample containing 1-3 micrograms of *N*-methylnicotinamide to 15 ml. Add 5 ml. of freshly prepared refrigerated 35% urea solution and 10 ml. of methyl ethyl ketone. Mix well and add 2 ml. of 40% sodium hydroxide solution. Mix, and set aside for 4 minutes. Add 4.5 ml. of 1:1 hydrochloric acid and shake. Add about 1 gram of monopotassium phosphate. Heat at 75-78° for 3 minutes. Cool to $23 \pm 0.5^\circ$.

Treat the blank similarly but add the methyl ethyl ketone after the monopotassium phosphate. Read the fluorescence of the lower phase in a Coleman 12C photofluorometer with filters B₁ and PC-1.

NIKETHAMIDE, *N,N*-DIETHYLNICOTINAMIDE

Nikethamide, the diethylamide of nicotinic acid, produces a pink color with cyanogen bromide and barbituric acid. By the first method given, the pink color is read at 560 m μ and Beer's law is followed up to 0.01 mg. per ml. This method is highly specific. A second method, a modification of the first, is more rapid and slightly less specific. In this modification, the color is read at 505 m μ and Beer's law is followed for 0.001-0.0035 mg. per ml.¹²⁵

Procedure—Dissolve a weighed amount of cyanogen bromide in warm water at 40° under a hood. Allow to come to room temperature and dilute to a 10% solution. Store in the refrigerator and use at room temperature. To prepare the saturated barbituric acid buffered solution, add 100 ml. of 3% monobasic potassium phosphate solution to 2 grams of barbituric acid. Shake vigorously at intervals for 1 hour and filter. To prepare the nikethamide standard solution, dilute an aliquot of nikethamide 25% standard with 0.3% monobasic potassium phosphate solution to 0.003 mg. per ml.

To 1 ml. of the standard solution containing 0.003 mg. of nikethamide per ml. and 1 ml. of sample of approximately the same concentration, add 0.5 ml. of the cyanogen bromide reagent. Mix, stopper, and let stand for 10 minutes. Heat at 50° for 25 minutes and then store at 20° for 5 minutes. Read at 560 m μ against a standard blank in which the cyanogen bromide is replaced by water.

¹²⁵ O. Pelletier and J. A. Campbell, *J. Pharm. Sci.* **51**, 594-5 (1962); cf. Rosa C. D'Alessio de Carnevale Bonino, *Publ. Inst. Invest. Microquim., Univ. Nac. Litoral* (Rosario, Arg.) **24**, No. 22, 83-93 (1958).

ETHIONAMIDE, 3-ETHYLISOTHIONICOTINAMIDE

Ethionamide is determined by its reaction with osmium tetroxide.¹²⁶ The reaction conforms to Beer's law over the range of 5-60 mg. per liter. Absorption maxima are at 270 and 430 m μ .

Procedure—Tablets. Digest the powdered sample equivalent to about 0.25 gram of ethionamide with 1:175 sulfuric acid. Filter, and wash the residue with the 1:175 sulfuric acid. Dilute to 500 ml. with acid of that strength. Mix 1 ml. of 1:16 sulfuric acid with 0.5 ml. of 7.5% osmium tetroxide solution in 0.8% sodium hydroxide solution. Add a portion of the sample solution and dilute to 10 ml. with water. After 5-20 minutes, read at 430 m μ against water.

MALEIC HYDRAZIDE, 1,2-DIHYDRO-3,6-PYRIDAZINEDIONE

Hydrazine, produced by the reduction of maleic hydrazide with zinc, forms a yellow color when heated with *p*-dimethylaminobenzaldehyde in acid solution.¹²⁷ All zinc must be removed from a flask previously used for distillation, since the presence of residual zinc leads to premature destruction of the maleic hydrazide. This method has been successfully used to determine 0.01 to 0.2 mg. of maleic hydrazide in apples, apricots, cranberries, cranberry sauce, grass roots, lemon juice, lemon peel, onions, peaches, potatoes, potato chips, dehydrated potatoes, and wood dust.

Procedure—Food. Grind the sample to a soup-like consistency in a high-speed blender, adding water if necessary. To remove volatile basic interferences, transfer 2 grams of the sample to the 300-ml. distillation flask, as shown in Figure 12. Dry the socket neck joint and add 50 grams of sodium hydroxide pellets, 1 ml. of refined vegetable oil as an anti-foaming agent, and 40 ml. of water. Add 1 ml. of high-boiling-point oil to the thermometer well, and insert the thermometer. Heat the flask on a high-temperature hot plate, and swirl gently every 20 seconds until the sodium hydroxide dissolves and gentle boiling starts. When the tempera-

¹²⁶ G. Popa, I. C. Ciurea, C. Lazar, and C. Cristesen, *An. Univ. Bucuresti, Ser. Stiint. Nat. Chim.* **11**, 91-5 (1962).

¹²⁷ Paul R. Wood, *Anal. Chem.* **25**, 1879-83 (1953); J. R. Lane, D. K. Gullstrom and J. E. Newell, *J. Agr. Food Chem.* **6**, 671-4 (1958); I. Hoffman, *J. Assoc. Offic. Agr. Chem.* **44**, 723-5 (1961); J. R. Lane, *J. Assoc. Offic. Agric. Chemists* **46**, 261-8 (1963).

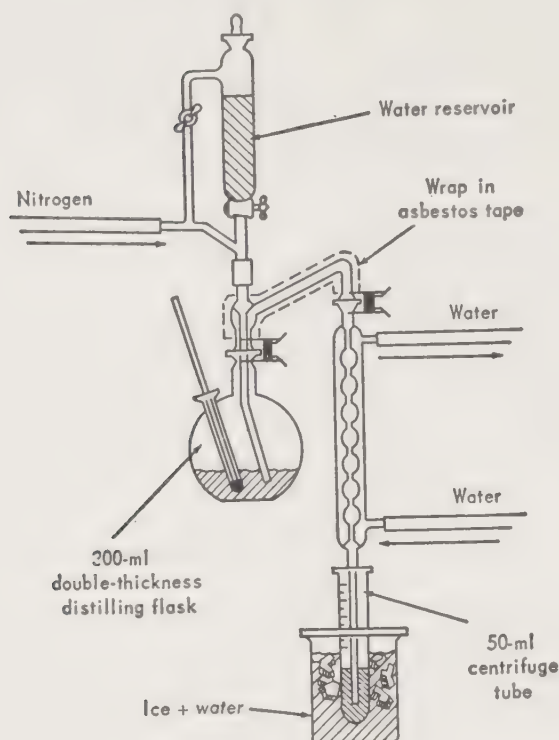


FIG. 12.

Distillation apparatus for maleic hydrazide

ture reaches 160° , remove the flask and let it cool for 5 minutes. Wipe the socket joint clean and dry. Add 0.5 gram of ferrous chloride and 5 grams of 10-mesh zinc.

Quickly grease the socket joint with a light film of high-vacuum silicone grease and attach the flask to the apparatus. Center the flask firmly on an asbestos pad. Place 4 ml. of *p*-dimethylaminobenzaldehyde reagent in the 50-ml. centrifuge tube in the apparatus and immerse the condenser tip. Adjust the flow of dry nitrogen to 3 bubbles per second in the receiver. Adjust the water in the condenser to flow rapidly. Heat the flask with a Bunsen burner, centering the tip of the outer cone of the flame on the asbestos pad. When boiling starts, adjust the distance of the burner so that the foaming contents fill approximately 75% of the flask. Distil until the temperature reaches 173° . Slowly add water from the reservoir until the temperature drops to 168° . Turn off the water and distil until the temperature rises again to 173° . Continue the addition of water and distillation to the temperatures of 168° and 173° , respectively, until 40 ml. is collected in the receiver. Remove the receiver. If, during

distillation, the solution in the receiver becomes turbid or a precipitate appears, add 2 drops of concentrated sulfuric acid and shake.

Read at 430, 460, and 490 $m\mu$ against 4 ml. of *p*-dimethylaminobenzaldehyde reagent diluted to 40 ml. as a blank.

Plant and animal tissue. Finely grind and blend a sample of not over 300 grams. Added water may be necessary to facilitate this. Take a sample equivalent to 0.5-3 grams of the original material, toward the lower limit if the starch content is high. Add to the flask of the apparatus (Fig. 12). Wash in with 15 ml. of water and add 30 grams of sodium hydroxide. Heat to 180° to minimize interferences.

Place 1 ml. of oxygen-free water and one drop of concentrated sulfuric acid in the receiver. Immerse the condenser tube. Add 15 grams of 30-mesh granular zinc to the flask. Put in place and pass nitrogen to remove the oxygen from the system. A bubble rate of 100-150 per minute in the receiver is appropriate.

Heat to the distillation temperature, over 140°, as rapidly as possible, using a free flame, usually within 1-2 minutes. When the temperature reaches 180°, add 10 ml. of oxygen-free water slowly through the dropping funnel. Again raise to 180°. The distillation should not require over 15 minutes. During the distillation, check the receiver with pH paper to make sure it remains acid.

If experience has shown that an interfering pink color develops in the receiver, apply a benzene extraction. To the contents of the receiver, add one drop of concentrated sulfuric acid for each 5 ml. of distillate. Shake with 10, 10, and 10 ml. of benzene, discarding the washings. Bubble nitrogen through the distillate to remove dissolved benzene. Heating to drive off dissolved benzene will cause low results.

As reagent, dissolve 0.2 gram of *p*-dimethylaminobenzaldehyde in 5 ml. of 1:17 sulfuric acid. Add 0.5 ml. of this to 5.5 ml. or less of the distillate and adjust the volume to 6 ml. If extra acid has been added for benzene extraction, add 10% potassium hydroxide solution, dropwise, until a precipitate of reagent just persists. Redissolve with 1:35 sulfuric acid before adjusting to the 6-ml. volume. After 15 minutes, read at 455 $m\mu$ against a reagent blank. The reagent may alternatively have been in the receiver, and so used, shows lack of acidity by precipitating.

Tobacco. Prepare dilute hydrochloric acid by dilution of 270 ml. of concentrated acid to 1 liter. Add 50 ml. of this to 1 gram of sample in a 300-ml. lipped conical flask. Add some paraffin wax to control foaming

and bring to a moderate boil. Occasionally wash down the sides of the container. When reduced to 20 ml., wash down the walls to give about 50 ml. Again boil, until the volume is substantially reduced. Add 50 ml. of 7% sodium hydroxide solution slowly with stirring. Boil down to about 20 ml. Let cool, rinse down the walls with about 30 ml. of water, and concentrate again. Repeat once more.

Transfer with a minimum of water to the distilling apparatus shown in Figure 12. Put 4 ml. of 1:35 sulfuric acid in the receiving cylinder, with the delivery tube below its surface. Deliver steam rapidly without the distilling flask being in place. Heat the wax bath to $200 \pm 10^\circ$. Add 15 grams of zinc to the distilling flask and put in place. Position the wax bath around the flask. Distil until about 20 ml. has been collected. Remove the receiver and the wax bath. Without stopping the passage of steam, remove the distilling flask and empty at once to prevent the caustic soda from solidifying. To prepare for the next run, rinse the condenser and delivery tube successively with petroleum ether, ethanol, water, concentrated sulfuric acid, and water.

Filter the distillate through a medium-porosity fritted funnel and evaporate to about 6 ml. Add 2 ml. of 1% dimethylaminobenzaldehyde in 1:35 sulfuric acid. Dilute with washings to 10 ml. After 15 minutes, read at 455 $m\mu$ against a blank of untreated tobacco.

1-HYDRAZINOPHTHALAZINE

The attached hydrazine group of 1-hydrazinophthalazine forms a highly colored complex upon reaction with ninhydrin in an acid medium.¹²⁸ 1-Hydrazinophthalazine also reacts with sulfhydryl and carbonyl radicals, glucose, and heavy metal ions. Sodium, potassium, magnesium, calcium, caesium, and barium do not react with the drug, whereas ferric, cupric, manganous, and cobaltous ions do. The ferric ion reacts, but the ferrous ion does not. The following drugs also have similar absorption spectra with the reagent: 1-hydrazino-4-methylphthalazine, 1,4-dihydrazinophthalazine, and 3-hydrazino-6-phenyl-1,2-diazine. The color with ninhydrin is stable for 50-100 hours and Beer's law is followed up to 100 micromoles of 1-hydrazinophthalazine.

Procedure—Urine. Adjust the pH of a 100-ml. aliquot of the sample to 3 and the ionic strength to 0.5 with glacial acetic acid. When necessary, add sodium acetate. Add 0.5 ml. of 2.4% ninhydrin solution in isopropanol.

¹²⁸ H. Mitchell Perry, Jr., *J. Lab. Clin. Med.* **41**, 566-73 (1953).

Heat at 70° for 7 minutes. Cool immediately in ice and extract with vigorous shaking for at least 30 seconds with 10 ml. of chloroform. After the layers have partially separated, clear the yellow ninhydrin-hydrazinophthalazine layer by centrifuging. Read at 460 m μ . If the solution is too opaque to read, dilute with chloroform.

ISONIAZID, ISONICOTINIC ACID HYDRAZIDE

At a controlled pH, isonicotinic acid hydrazide reacts with a vanillin solution to produce a yellow aldimine, 1-isonicotinyl-2-(3-methoxy-4-hydroxy-benzylidene)-hydrazine.¹²⁹ The proteins cannot be precipitated by tungstic acid, since this acid interferes. There is also interference from *p*-aminosalicylic acid, which reacts with the reagent. Streptomycin, dihydrostreptomycin, and viomycin do not interfere. Beer's law is followed for 0.02-0.1 mg.

After extraction of the isonicotinic acid hydrazide from blood samples using carbon tetrachloride, the hydrazide is developed with methyl orange.¹³⁰ Ascorbic acid, aminopyrine, and sugars add to the extinction, and *p*-aminosalicylic acid reduces the extinction. Isonicotinic acid, nicotinic acid, and vitamin B₁ do not interfere. *p*-Aminosalicylic acid can be extracted from a solution in 1:100 hydrochloric acid with ether, and the isoniazid may then be read directly at 255 and 305 m μ .¹³¹

Isonicotinic acid hydrazide in serum is separated by means of an ion-exchange column. Then a fluorescent compound is formed with hydrogen peroxide by heating at a neutral pH.¹³² Neither the acetyl derivative of isonicotinic acid hydrazide nor isonicotinic acid interferes. Interference from nicotinamide is eliminated by means of a serum blank. The isopropyl derivative of isonicotinic acid hydrazide interferes. Other pyridine derivatives yield highly fluorescent products with hydrogen peroxide and heat but can be corrected by the blank.

Cupric ions in the presence of acetone produce a color that follows Beer's law for 0.1-1.7 mg. of isonicotinic acid hydrazide per ml.¹³³

¹²⁹ Miloche Yantchitch, *Ann. Biol. clin.* **12**, 328-9 (1954); Edward N. Deeb and Guy R. Vitagliano, *J. Am. Pharm. Assoc.* **44**, 182-5 (1955); M. Bracco and E. Savio, *Ann. Med. Sondalo* **9**, 201-5 (1961).

¹³⁰ Yahyoe Kinoshita and Shigetaka Moriyama, *Bull. Nagoya City Univ. Pharm. School No. 1*, 37-9 (1953).

¹³¹ M. C. Dutt and T. H. Chua, *J. Pharm. Pharmacology* **16**, 696-9 (1964).

¹³² M. T. Hedrick, J. W. Rippon, L. E. Decker and J. Bernsohn, *Anal. Biochem.* **4**, 85-98 (1962).

¹³³ S. Zommer and T. Lipiec, *Acta Polon. Pharm.* **20**, 229-32 (1963).

The alkaline hydrolysis of 4-pyridylpyridinium chloride produces glutaconic aldehyde, which forms a stable yellow color upon reaction with isonicotinic acid hydrazide.¹³⁴ Beer's law is followed for 0.002-0.02 mg. and the acetylated form of the hydrazide does not interfere.

2,3-Dichloro-1,4-naphthoquinone is a sensitive reagent for isonicotinic acid hydrazide.¹³⁵ Thiacetazone, pyrazinamide, 4-aminosalicylic acid, sulfanilamide, and chloranil may be present.

Sodium β -naphthoquinone-4-sulfonate combines with the hydrazide portion of isonicotinic acid hydrazide to produce an orange-red color with an absorption maximum at 480 m μ ¹³⁶ (see Vol. IV, pp. 259-61). The following do not interfere: acetamide, acetanilide, picolinic acid, ethylnicotinate, and pyridine. The following form colored compounds with the reagents, with the absorbance maximum in m μ noted: hydrazide sulfate, 440-450 m μ ; phenylhydrazine hydrochloride, 440 m μ ; aniline hydrochloride, 445 m μ ; hydroxylamine hydrochloride, 440 m μ ; urea, 480 m μ . Semicarbazide forms the same color with the reagent but has considerably less absorption than an equimolar quantity of hydrazide. Hydroxylamine reacts with the reagent to produce a compound with an absorption maximum at 440 m μ . Beer's law is followed for 0.02-0.1 mg.

The reaction of isonicotinic acid hydrazide with 2-bromo-1-acetonaphthone forms a pyridinium bromide that is colored in basic solution.¹³⁷ 2-Picoline and picolinic acid do not interfere. In the presence of isonicotinic acid, nicotinic acid, nicotinamide, 3-picoline, or 4-picoline, the isonicotinic acid hydrazide is read at 500 m μ instead of the usual 470 m μ .

Another reagent for isonicotinic acid hydrazide is 4-fluoro-1,3-dinitrobenzene.¹³⁸ Although 4-aminosalicylic acid gives a color with the reagent, the reaction is more sensitive for isonicotinic acid hydrazide, and the separation of 4-aminosalicylic acid, as described in the procedure, results in errors of less than $\pm 0.5\%$. An alcoholic solution of *p*-dimethylamino-benzaldehyde is a reagent for isonicotinic acid hydrazide.¹³⁹ Hydrazine

¹³⁴ Benjamin Prescott, Gladys Kauffmann and Walter D. James, *Proc. Soc. Exptl. Biol. Med.* **84**, 704-6 (1953); Benjamin Prescott, Sol Katz and Gladys Kauffmann, *J. Lab. Clin. Med.* **44**, 600-3 (1954).

¹³⁵ Masami Akatsuka, *J. Pharm. Soc. Japan* **83**, 227-233 (1963).

¹³⁶ E. L. Pratt, *Anal. Chem.* **25**, 814-6 (1953).

¹³⁷ Shigo Hirose and Toyozo Uno, *J. Pharm. Soc. Japan* **81**, 1623-5 (1961).

¹³⁸ K. C. Agrawal and B. N. Dutta, *Indian J. Appl. Chem.* **24**, 186-192 (1961).

¹³⁹ Ichiroemon Kidani, Tatsumi Nakashima, Yoshimoto Koichi and Shizuka Kasahara, *Bull. Natl. Hyg. Lab.* No. 72, 95-7 (1954); G. Machek, *Scientia Pharm.* **24**, 11-17 (1956); Norman F. Poole and Arthur E. Meyer, *Proc. Soc. Exptl. Biol. Med.* **98**, 375-7 (1960).

sulfate forms a colored substance with a maximum absorption at 480 $m\mu$ and a lesser absorption at 409 $m\mu$. Amines, amino acids, and urea give a similar reaction.

A yellow chromogen results from the reaction between isonicotinic acid hydrazide and sodium pentacyanoaminoferroate, $\text{Na}_3[\text{Fe}(\text{CN})_5\text{NH}_3]$, with a maximum absorption at 430 $m\mu$.¹⁴⁰ Beer's law is followed, and as low as 0.001 mg. per ml. can be measured. The reaction is not affected by temperature. The optimum pH is 5-7.

A saturated aqueous solution of 1,2,4-aminonaphthol sulfonic acid is a reagent for isonicotinic acid hydrazide.¹⁴¹ After hydrolysis to the acid, isonicotinic acid hydrazide is determined with cyanogen bromide.¹⁴² Beer's law is followed for 0.001-0.025 mg. per ml.

Isoniazid reduces ferricyanide quantitatively to ferrocyanide. By adding ferric ion, phosphoric acid, and a dispersing agent, it is read colorimetrically.¹⁴³ The reducing action of isoniazid can be applied to sodium phosphomolybdate at pH 6.5-7.2 to produce molybdenum blue.¹⁴⁴

Isoniazid is readily hydrolyzed by aqueous sodium hydroxide to hydrazine. The latter forms a fluorescent aldazine with 2-hydroxy-1-naphthaldehyde. The maximum fluorescence is at 534 $m\mu$ and is linear over the range of 0.02-0.8 microgram per ml. of hydrazine, corresponding to 0.004-0.012 mg. of isoniazid.¹⁴⁵ Isoniazid and ascorbic acid are read in the presence of nicotinamide, using two wave lengths in the ultra-violet.¹⁴⁶

Isoniazid and acetylisoniazid are extracted from 2-3 ml. of blood, serum, or urine.¹⁴⁷ The sample is first saturated with ammonium sulfate. It is then extracted with 1:9:10 heptane-ethylene dichloride-isobutanol. The materials are extracted from the solvent mixture with 1:350 sulfuric

¹⁴⁰ Takio Naito, Hideaki Shirai and Noriichi Oda, *Bull. Nagoya City Univ. Pharm. School* No. 3, 34-7 (1955); Vincenzo Scardi, *Clin. Chim. Acta* 2, 134-9 (1957); *Arch. ital. sci. farmacol.* 7, 206-11 (1957); Vincenzo Scardi and V. Bonavita, *Clin. Chim. Acta* 4, 161-4 (1959).

¹⁴¹ Sergio A. Gomez, *Arch. bioquim. quim. y farm. Tucuman.* 8, 69-95 (1957-58).

¹⁴² A. Defranceschi and V. Zamboni, *Ciorn. biochim.* 1, 405-16 (1952); J. F. M. Holscher, *Minerva pediat.* 7, 160-1 (1955); J. H. Peters, *Am. Rev. Respirat. Diseases* 81, 485-97 (1960).

¹⁴³ P. Blanc, P. Bertrand and L. Liandier, *Lyon pharm. Spec.* No. 7, 161-4 (1956).

¹⁴⁴ Tomoyuki Akiyama, Takako Yabuchi and Katsuyo Shiono, *Kyoto Yakku Daigaku Gakko* 7, 48-56 (1959).

¹⁴⁵ Tsutomu Momose, Yo Ueda, Yoshiko Mukai and Kyoko Watanabe, *J. Pharm. Soc. Japan* 80, 225-8 (1960).

¹⁴⁶ G. Machek and F. Lorenz, *Sci. Pharm.* 30, 25-37 (1962).

¹⁴⁷ Q. C. Belles and M. L. Littleman, *Anal. Chem.* 32, 720-1 (1960).

acid. When read in this aqueous acid solution, their maximum absorbance is at 265 m μ . At pH 10, the maximum absorbance of the acetyl derivative is at 300 m μ , but that of isoniazid is unchanged. Both shift to 300 m μ at pH 12. Isonicotinic acid makes no shift with pH. They are separable by chromatography on paper saturated with 0.2 M phosphate buffer for pH 6.0 and dried. The solvent is butanol 78: butadine 20: salicylaldehyde 2, saturated with the phosphate buffer. After the spots are located with 1:110 hydrochloric acid saturated with salicylaldehyde, it is dried to evaporate the aldehyde, and the spots are extracted with 1:350 sulfuric acid.

Isoniazid, its derivatives and metabolites are cleaved in the pyridine ring with cyanogen chloride.¹⁴⁸ The glutacondialdehyde so formed condenses with barbituric acid to give a polymethine dye with a maximum absorption at 600 m μ . As little as 0.05 microgram of isoniazid per ml. can be detected.

The yellow color of isoniazid and sodium pentaacyanoaminoferroate is read after deproteinizing with metaphosphoric acid.¹⁴⁹ Isoniazid gives a yellow color on heating with ethylenic dicarboxylic acids when made alkaline.¹⁵⁰ Applicable acids are maleic, fumaric, citraconic, mesaconic, itaconic, and *cis*- and *trans*-aconitic acid. Saturated acids and other pyridine derivatives give no color.

Isoniazid is coupled with salicylaldehyde and then extracted.¹⁵¹ As a general reaction, a hydrazide condenses with ninhydrin. Heating eliminates water and rearrangement gives an azo-type double bond. Reduction with ascorbic acid cleaves this bond to yield an amine and an amide. The amine then condenses with another mole of ninhydrin to give Ruhemann's purple.¹⁵² For rapid determination *trans*-cinnamaldehyde is used.¹⁵³ The blank on serum is high and hydrazine sulfate interferes.

Procedure—*By vanillin. Oxalated blood plasma or serum.* Dilute a 4-ml. sample to 8 ml. with water and add 8 ml. of 20% trichloroacetic acid solution. Stopper, shake vigorously, and centrifuge at 1500 rpm. for 5 minutes. Filter through a retentive paper.

¹⁴⁸ Walter Nielsch and Lieselotte Gießer, *Arzneimittel-Forsch.* **9**, 636-41, 700-7 (1959).

¹⁴⁹ Vincenzo Scardi and Vincenzo Bonavita, *Clin. Chem.* **3**, 728-31 (1957).

¹⁵⁰ E. Neuzil and Y. LeDue, *Bull. Soc. Pharm. (Bordeaux)* **100**, 159-79 (1961).

¹⁵¹ J. Guillaume, A. Tacquet, and J. Y. Berthelot, *Rev. franç. études clin. et bio.* **5**, 729-33 (1960).

¹⁵² Akio Tsuji and Masami Hojo, *Bunseki. Kagaku* **11**, 1255-62 (1962).

¹⁵³ L. Eidus and E. J. Hamilton, *Clin. Chem.* **10**, 581-8 (1964).

As a reagent, dissolve 2 grams of vanillin in 25 ml. of 95% ethanol and dilute to 100 ml. with water. Add 1 ml. of reagent to an 8-ml. protein-free aliquot containing 0.02-0.1 mg. of isonicotinic acid hydrazide. Add 1 ml. of 1:35 sulfuric acid and mix by swirling. Let stand for 10 minutes. Read at 400 $m\mu$ against a reagent blank containing 8 ml. of water in place of the sample.

By methyl orange. Blood. Dilute a 1-ml. sample to 10 ml. with water. Add 2 ml. of 10% sulfuric acid and 2 ml. of 10% sodium tungstate solution. Centrifuge to remove protein and decant the supernatant liquid. Wash the precipitate with two 5-ml. portions of water and add the washings to the supernatant layer. Shake with 1 ml. of 1% potassium bromate solution and 5 ml. of carbon tetrachloride. Separate the carbon tetrachloride layer and wash the residue with two 5-ml. portions of carbon tetrachloride. To the combined carbon tetrachloride extracts, add 10 ml. of 1% methyl orange solution, shake, and read the aqueous layer.

By hydrogen peroxide. Serum. Add a 2-ml. sample to 2 ml. of 10% zinc sulfate septahydrate, and 14 ml. of water. After 5 minutes, add 2 ml. of 2.3% sodium hydroxide solution and centrifuge. To a 15-ml. portion of the supernatant liquid, add 0.1-0.2 ml. of a 5% solution of the disodium salt of ethylenediaminetetraacetic acid to complex any zinc left in the sample after protein precipitation. Adjust the pH to 6.5 with 1-3 drops of 0.4% sodium hydroxide solution. To prepare the cation-exchange column, stir Amberlite XE-64, in the hydrogen form, in water for 20 minutes and allow to settle for 3 minutes. Decant. Repeat this procedure 5 times to remove most of the finer particles. As a column, use a tube 5-7 mm. in diameter, fitted with short lengths of latex tubing and small screw clamps. Avoid polyethylene. Add the water suspension of the resin to the column until the resin bed is 20-30 mm. in depth. Wash the column with 1 ml. of 2:3 hydrochloric acid to remove any substances in the resin blank, that might cause fluorescence, and wash with water until chloride-free. Allow the sample to flow through the column at a rate of 10-12 drops per minute. Rinse the resin with 3 ml. of water and discard the effluent. Elute isonicotinic acid hydrazide with 1 ml. of 1:15 hydrochloric acid followed by two 1-ml. water washes. Adjust the pH of the eluate to 7 with approximately 0.6 ml. of 4% sodium hydroxide solution.

Prepare a buffer for pH 8.7 from a 12.1% solution of *tris*(hydroxymethyl)aminomethane and 1:1 hydrochloric acid. To the eluate, add 0.1

ml. of the buffer and dilute to 5 ml. with water. To a 2-ml. aliquot placed in a Folin-Wu sugar tube, add 0.2 ml. of cold 30% hydrogen peroxide and heat at 100° for 30 minutes. Let stand in cold water for 15 minutes and read the fluorescence at 415 $m\mu$ within 1 hour by activation at 320 $m\mu$. In order to obtain the maximum fluorescence, the pH should be between 6.5 and 7.5 after oxidation.

By 4-pyridylpyridinium dichloride. Plasma. Treat a 2-ml. citrated sample with 6 ml. of water and 2 ml. of 15% trichloroacetic acid. Filter. Treat 2 ml. of the filtrate successively with 0.5 ml. of 1% 4-pyridylpyridinium dichloride solution, 0.7 ml. of 8% sodium hydroxide solution, and 0.5 ml. of 1:5 hydrochloric acid. Dilute to 5 ml. and read after 15 minutes at 425 $m\mu$ against water. The color is stable for at least an hour.

By sodium pentacyanoaminoferroate. Serum or plasma. Shake a 1-ml. sample with 2 ml. of water. Add 1 ml. of glacial acetic acid and mix. Heat at 100° for 2-3 minutes and centrifuge for 10 minutes at 3000 rpm. To 2 ml. of the supernatant layer, add 1.5 ml. of McIlvaine buffer for pH 6.5 and 0.5 ml. of 0.2% sodium pentacyanoaminoferroate solution in 0.02N ammonium hydroxide. Read at 430 $m\mu$ after 10 minutes.

Tissue. To prepare 0.2% sodium pentacyanoaminoferroate solution, add 32 ml. of concentrated ammonium hydroxide to 10 grams of powdered sodium nitroprusside. Wash the precipitate several times with 95% ethanol. Filter, and wash with ether. Store in a dessicator.

Prepare a homogenate 1:5 in 0.9% sodium chloride solution. Shake 1 ml. of the homogenate with 1 ml. of water and 1 ml. of 20% metaphosphoric acid solution, and allow 10 minutes for the protein to flocculate at room temperature. Centrifuge for 10 minutes. To 2 ml. of the supernatant fluid, add 0.5 ml. of 16.5% diammonium phosphate solution. Mix well and add 0.5 ml. of sodium pentacyanoaminoferroate reagent. After 10 minutes, read at 430 $m\mu$ against a reagent blank.

Liver. Follow the procedure for tissues, diluting the sample 1:10. Use a 2-ml. homogenate and 2 ml. of metaphosphoric acid reagent. Combine 2 ml. of the supernatant layer with 0.5 ml. of 33% diammonium phosphate.

By cyanogen bromide. Serum and cerebrospinal fluid. To prepare the reagent, pour 40 grams of bromine into 300 ml. of water and cool in ice.

Decolorize by dropwise addition of 10% sodium cyanide solution and dilute to 500 ml. with water. As a buffer, add 1 volume of 0.8% sodium hydroxide solution to 5 volumes of 2.8% dibasic sodium phosphate solution.

To a 4-ml. sample, add 2 ml. of 20% trichloroacetic acid solution and 4 ml. of water. Stir, warm at 60-70° for 10 minutes, cool, and centrifuge for 10 minutes. To 5 ml. of the supernatant liquid, add 1.5 ml. of 50% sodium hydroxide solution. Heat for 1 hour at 100°, cool, and add 3.5 ml. of 1:1 hydrochloric acid. Neutralize with 1:5 hydrochloric acid or 10% sodium hydroxide solution. Dilute to 10 ml., centrifuge, and add 2 ml. of buffer. After 30 minutes, recentrifuge. To 6 ml. of the supernatant liquid, add 3 ml. of cyanogen bromide reagent. Read at 427 m μ against a blank. Add 1 ml. of cyanogen bromide reagent to the sample and blank and let stand for 15-25 minutes. Read at 427 m μ and calculate from the difference between the two readings.

Urine. Follow the preparation of the reagent and buffer as described under "Serum and cerebrospinal fluid." Dilute a sample 1:10. To a 2-ml. aliquot, add 2 ml. of 8% sodium hydroxide solution. Heat at 100° and add 4-5 drops of 2.5% potassium permanganate solution every 5 minutes until decolorized. After 1 hour, add 0.5 ml. of 5% formic acid solution. Stir to precipitate manganese, and cool. Dilute to 8 ml., stir, and centrifuge for 5 minutes. To 4 ml. of the supernatant liquid add 1:5 hydrochloric acid until neutralized, usually about 0.75 ml. Dilute to 5 ml. with water. Add 4 ml. of reagent and read at 427 m μ . Complete by the procedure under "Serum and cerebrospinal fluid," starting at "Add 1 ml. of cyanogen bromide reagent to the sample . . ."

Alternatively, shake a 2-ml. urine sample with 8 ml. of water. To a 4-ml. aliquot, add 2 ml. of 20% trichloroacetic acid solution and 4 ml. of water. Shake and warm at 60-70° for several minutes. Cool and centrifuge. Heat 5 ml. of the supernatant liquid with 1.5 ml. of 50% sodium hydroxide solution at 100° for 1 hour. Cool, and add 3.5 ml. of 1:1 hydrochloric acid. Complete the neutralization with 1:5 hydrochloric acid or 10% sodium hydroxide solution. Dilute to 10 ml. Add charcoal, shake, and filter. Add 2 ml. of the phosphate buffer and 1 ml. of 5% cyanogen bromide reagent. After 30 minutes, read at 425 m μ .

By 4-fluoro-1,3-dinitrobenzene. *Pharmaceutical preparations containing 4-aminosalicylic acid or its calcium salt.* Dissolve a sample equivalent to 10 mg. of isonicotinic acid hydrazide in water. Filter, and dilute to

50 ml. Dilute a 5-ml. aliquot to contain 0.02 mg. of isonicotinic acid hydrazide per ml. If the calcium salt of 4-aminosalicylic acid is present, add concentrated hydrochloric acid to the filtrate and allow the precipitate to settle before filtering. Adjust a 5-ml. aliquot of the filtrate to pH 7 with 20% sodium hydroxide solution and dilute to 50 ml. Shake a 4-ml. aliquot with 6 ml. of water, 3 ml. of 5% sodium borate solution, 5 ml. of isopropanol, and 2 ml. of 1.5% 4-fluoro-1,3-dinitrobenzene solution in ethanol. After 20 minutes, read at 520 $m\mu$.

Serum. Deproteinize serum by adding 4 volumes of 95% ethanol. Centrifuge, and take a 5-ml. sample of the clear-layer equivalent to 1 ml. of serum. Add 0.5 ml. of 0.4% solution of 1-fluoro-2,4-dinitrobenzene and 0.1 gram of borax. Heat for 5 minutes at 80°, cool, and read at 500 $m\mu$.

By cupric ion. Dissolve a sample containing 2.5-40 mg. of isonicotinic acid hydrazide in a small volume of water. Add 5 ml. of acetone and 3 ml. of 2.4% cupric nitrate solution. Dilute to 25 ml. with water and read.

By 2,3-dichloro-1,4-naphthoquinone. To a 3-ml. sample containing 0.001-0.01 mg. of isonicotinic acid hydrazide per ml. add 1 ml. of 0.03% 2,3-dichloro-1,4-naphthoquinone solution in ethanol or methanol, and 1 ml. of 0.1% potassium carbonate solution. Let stand for 10 minutes and read at 620 $m\mu$.

By β -naphthoquinone-4-sulfonate. Dissolve 40 mg. of recrystallized sodium β -naphthoquinone-4-sulfonate and 250 mg. of sodium sulfite in 100 ml. of water. Add 3 ml. of 6% acetic acid solution and dilute to 200 ml. with water. Dilute a sample containing 0.02-0.1 mg. of isonicotinic acid hydrazide to 3 ml. with water. Add 10 ml. of reagent and 2 ml. of 8% sodium hydroxide solution. Invert to mix and let stand for 15 minutes. Read against a reagent blank at 480 $m\mu$.

By bromo-1-acetonaphthone. Reflux 1 ml. of an ethanolic solution containing 0.001-0.04 mg. of isonicotinic acid hydrazide and 0.5 ml. of 0.25% 2-bromo-1-acetonaphthone solution in ethanol for 25 minutes. Add 1 ml. of 0.1% sodium ethoxide solution in ethanol and dilute to 5 ml. with 95% ethanol. Read at 470 $m\mu$ after 3 minutes. In the presence of 0.015 mg. of isonicotinic acid, 0.03 mg. of nicotinic acid, 0.03 mg. of nicotinamide, 0.2 mg. of 3-picoline, or 0.2 mg. of 4-picoline, isonicotinic acid hydrazide in the range of 0.004-0.07 mg. is measured at 500 $m\mu$.

By *p*-dimethylaminobenzaldehyde. To a 2-ml. sample containing 0.1-0.2 mg. of isonicotinic acid hydrazide, add 2 ml. of 2% *p*-dimethylaminobenzaldehyde solution in 95% ethanol and 3 ml. of isobutanol. Shake, let stand for 10 minutes, and add 1 ml. of 1% oxalic acid. Read at 409 $m\mu$.

Alternatively, prepare a reagent by dissolving 400 mg. of *p*-dimethylaminobenzaldehyde, 2 ml. of 1:9 hydrochloric acid, and 1 ml. of 0.5% sodium nitrite solution in 100 ml. of methanol. Shake a powdered sample containing 100 mg. of isonicotinic acid hydrazide for 20 minutes with 50 ml. of warm methanol. Filter and dilute to 100 ml. Dilute a 5-ml. aliquot with 25 ml. of methanol. Add 5 ml. of reagent to a 5-ml. aliquot and read after 20 minutes at 525 $m\mu$.

By 1,2,4-aminonaphthol sulfonic acid. Dilute a 0.5-1.5-ml. sample to 5 ml. with water. Add 1 ml. of freshly prepared saturated aqueous solution of 1,2,4-aminonaphtholsulfonic acid, 1 ml. of butanol, 1 ml. of 0.4% sodium hydroxide solution, and 2 ml. of ethanol. Mix, let stand for 5 minutes, and read at 450-480 $m\mu$.

By *ninhydrin*. To 2 ml. of a solution of isoniazid containing about 0.04 mg. per ml., add 1 ml. of 0.05% ascorbic acid solution, 1 ml. of 1% *ninhydrin* solution, and 1 ml. of pyridine. Heat at 100° for 25 minutes and cool. Shake for 2 minutes and dilute to 25 ml. with water. Read at 570 $m\mu$.

*Direct reading.*¹⁵⁴ *Plasma or urine.* For use, wash isoamyl alcohol with three equal volumes of 1:3 hydrochloric acid, then with 10% sodium hydroxide solution, then with several portions of water. As the solvent, mix 1:4 with ether.

Mix 1-3 ml. of plasma or urine containing 0.003-0.05 mg. of isonicotinic acid hydrazide with 3.2 grams of diammonium sulfate. Add 1 ml. of 2% sodium hydroxide solution and 40 ml. of the mixed solvent. Shake 30 minutes for plasma, 10 minutes for urine. Centrifuge for 5 minutes. Take 30 ml. of the organic phase and add 4 ml. of 1:100 hydrochloric acid. Shake for 5 minutes and centrifuge for 3 minutes. Separate the acid extract and read at 266 $m\mu$.

Alternatively, take 1-3 ml. of the acid extract and add 0.5 ml. of 1:1 hydrochloric acid. As the reagent, dissolve 1.2 gram of *p*-dimethylaminobenzaldehyde in 20 ml. of absolute ethanol and add 2 ml. of concentrated

¹⁵⁴ Jacques M. Kelly and Raymond B. Poet, *Am. Rev. Tuberc.* 65, 484-5 (1952).

hydrochloric acid, prepared fresh daily. Add 1 ml. of reagent and mix. Heat at 100° for 45 minutes and cool. Dilute to an appropriate volume with 1:100 hydrochloric acid and read at 450 m μ against 1:100 hydrochloric acid.

By trans-cinnamaldehyde. Serum. Shake 4 ml. with 1 ml. of 40% trichloroacetic acid. After 5 minutes, centrifuge for 10 minutes. Filter, and mix 2 ml. of the filtrate with 1.5 ml. of 0.1% *trans*-cinnamaldehyde in absolute ethanol. Read at 352-354 m μ .

N,N'-DIISONICOTINOYL HYDRAZINE

In the presence of isonicotinic acid hydrazide and its metabolites, *N,N'*-diisonicotinoyl hydrazine is developed with pentacyanoaminoferroate.¹⁵⁵

Procedure—To a 5-ml. solution containing 0.005-0.1 mg. per ml. of *N,N'*-diisonicotinoyl hydrazine, add 0.5 ml. of freshly prepared 0.25% solution of pentacyanoaminoferroate. Heat for 15 minutes at 60° and read at 333 m μ .

GLUCURONAMIDE AND GLUCURONOLACTONE ISONICOTINOYL HYDRAZONE

Glucuronamide and glucuronolactone react at different rates with hydroxylamine to form hydroxamic acids for reading as the ferric complex.¹⁵⁶

Procedure—Mix 1 ml. of 1:2 hydrochloric acid and 1 ml. of 2.3% hydroxyammonium chloride. Add a 1-ml. sample containing 0.05-0.5 mg. of the test substances. After 20 minutes at 40°, chill in ice. Add 1.5 ml. of 1:2 hydrochloric acid, then 1 ml. of 35% ferric alum solution. Dilute to 10 ml. with water and read at 470 m μ . A nomogram is required for getting the results when both are present.

PICOLINIC ACID, 2,5-PYRIDINECARBOXYLIC ACID, DINICOTINIC ACID

In a slightly acid medium, 2,5-pyridinecarboxylic acid forms a stable yellow to orange-red chelate with ferrous ion, which follows Beer's law

¹⁵⁵ B. P. Lisboa, *Naturwissenschaften* **46**, 109 (1959).

¹⁵⁶ Tsukinaka Yamanaka, Shinichi Asai and Jiro Aoki, *Arch. Pract. Pharm., Japan* **22**, (1), 60-63 (1962).

at 415 $m\mu$ up to 12.5 mg. per 25 ml. of solution.¹⁵⁷ The reaction is believed to be reversible and proceeds to the formation of the chelate in the presence of excess ferrous ion and to the formation of the acid in the presence of excess hydrogen ion. The optimum pH of the reaction is 3.5 to 4. The method is accurate to $\pm 1.8\%$ and has a precision of at least 0.2%. Nitric acid, 5-ethyl-2-methylpyridine, and 3-pyridinecarboxylic acid may be present. When isolated by paper chromatography by 4:1:5 butanol-acetic acid-water, or by 1:1 butanol-methanol, the extracted picolinic acid is read at 264 $m\mu$.¹⁵⁸ Beer's law applies at 2.5-20 micrograms per ml.

Procedure—*Reactor effluent, the oxidation product of 5-ethyl-2-methylpyridine and nitric acid.* As a buffer, dissolve 15 grams of anhydrous sodium acetate in 50 ml. of water. Add 70 ml. of glacial acetic acid and dilute to 200 ml. with water. Adjust to pH 3.6 with either sodium acetate or acetic acid. To prepare the reagent, dissolve 7 grams of ferrous ammonium sulfate in 50 ml. of water. Filter into a flask containing 1 ml. of glacial acetic acid and dilute to 100 ml. with water. This reagent must be used within 4 hours of preparation.

Mark three 25-volumetric flasks as A, B, and C. Add 1 drop of 1% phenolphthalein solution in neutral 95% ethanol, 1 drop of 8% sodium hydroxide solution, 10 ml. of buffer, and 2 ml. of ferrous reagent to A, and dilute to 25 ml. with water. This is the reagent blank. Dilute a known weight or volume of the reactor effluent so that an aliquot contains not more than 12.5 mg. of 2,5-pyridine dicarboxylic acid. Pipet the same size aliquots into flasks B and C. Add 1 drop of 1% phenolphthalein solution in neutral 95% ethanol to each, and then 8% sodium hydroxide solution until a red color appears. Add 10 ml. of buffer. Add 2 ml. of the iron reagent to flask C and dilute both B and C to 25 ml. Mix. Flask B contains the sample blank, which corrects for the yellow to brown color of the sample. Read flasks B and C at 415 $m\mu$, using flask A to set the instrument for zero optical density.

Solid preparations. Weigh 0.1-0.5 gram of the sample. Add 80 ml. of water and warm at 100° until the crystals dissolve. Addition of small amounts of 4% sodium hydroxide solution may aid solubilization. Cool

¹⁵⁷ Kuang Lu Cheng and John A. Riddick, *Anal. Chem.* **26**, 536-8 (1954).

¹⁵⁸ Nagahiro Ogasawara, Kinjiro Tamari and Masanichi Suga, *J. Agric. Chem. Soc. Japan*, **35**, 1312-14 (1961).

and dilute to 100 ml. with water. Follow the procedure for reactor effluent, starting at "Mark three 25-ml. volumetric . . ."

IPRONIAZID, 1-ISONICOTINIC ACID-2-ISOPROPYLHYDRAZIDE

Several cyclic compounds containing a nitrogen atom in the ring and NH_2 or NH groups in a side chain give a color reaction with sodium nitritopentacyanoferroate.¹⁵⁹ Both nialamide and iproniazid form a yellow color with the reagent and are determined together by this reaction in serum samples. Precipitation of serum proteins should be avoided, since a large coprecipitation of iproniazid and nialamide results. Interfering drugs are removed by extracting the iproniazid into chloroform. The recovery by chloroform extraction depends on the pH, which should be 7-7.5. Since pyrazinamide and cycloserine are also extracted into the chloroform, they interfere. Nicotinic acid and isonicotinic acid do not interfere. The yellow color formed is unstable at room temperature but is stable for 10-20 minutes at 10° . Beer's law is followed and the method is sensitive for 0.005-0.01 mg. of iproniazid and nialamide per ml.

Reaction of molybdic acid with iproniazid in an acetone medium produces a stable reddish complex.¹⁶⁰ The absorption curve of the color shows two distinct maxima at $430\text{ m}\mu$ and at $535\text{ m}\mu$. In most cases, the absorbance at $430\text{ m}\mu$, which is almost twice that at $535\text{ m}\mu$, is used. However, in cases when the sample blank is very yellow, the red band at $535\text{ m}\mu$ is preferred. Beer's law is followed for 0.04-0.2 mg. of iproniazid at either $430\text{ m}\mu$ or $535\text{ m}\mu$. The procedure is sensitive to 0.01 mg. The color reaches a maximum within 5-10 minutes and remains stable for at least 3 hours. Maximum color development is achieved with hydrochloric acid between 2 and 3 *N*. Isonicotinic acid hydrazide does not produce a color with the reagent. Compounds of the following general structure will produce a color:



where R is alkyl or aryl and R' is pyridine.

If the terminal nitrogen is disubstituted, no color is produced under the conditions of the test. In the determination of iproniazid in syrup, the

¹⁵⁹ K. B. Bjornesjo and B. Jarnulf, *Scand. J. Clin. Lab. Invest.* **14**, 408-15 (1962).

¹⁶⁰ Remo J. Colarusso, Morton Schmall, Ernest G. Wollish and E. G. E. Shafer, *Anal. Chem.* **30**, 62-5 (1958).

interference of sorbitol, propylene glycol, glycerol, or corn syrup is overcome by using the internal standard technic.

Procedure—*By sodium nitritopentacyanoferroate. Serum.* Extract a 5-ml. sample for 5 minutes with 25 ml. of chloroform. Filter and shake a 20-ml. portion of the extract with 5 ml. of 1:59 hydrochloric acid.

To prepare the sodium nitritopentacyanoferroate reagent, immediately before use mix equal volumes of 4% sodium nitroprusside solution and 16% sodium hydroxide solution. To 3 ml. of the hydrochloric acid extract, add 1 ml. of 1:5 acetic acid and cool to 10°. Add 1 ml. of cold sodium nitritopentacyanoferroate reagent and read at 440 m μ against a reagent blank after 10 minutes.

Urine. Adjust a 20-ml. sample to pH 7-7.5 with 0.8% sodium hydroxide solution and dilute to 25 ml. with water. Extract a 20-ml. aliquot with 50 ml. of chloroform for 5 minutes. Filter and shake 40 ml. of the extract with 5 ml. of 1:59 hydrochloric acid. Develop as for serum, from "To prepare the sodium . . ."

By ammonium molybdate. Tablets and ampoules. To prepare the standard solution, dissolve 75 mg. of iproniazid phosphate in water and dilute to 250 ml.

Weigh a sample equivalent to about 50 mg. of iproniazid base. Shake well to dissolve in water. Dilute to 250 ml. with water and allow any excipients to settle. Centrifuge or filter if necessary.

Mark three small flasks with narrow necks as blank, standard, and sample. To the blank, add 1 ml. of water. To the standard, add 1 ml. of iproniazid standard solution. To the sample, add 1 ml. of sample solution. Into each, add 10 ml. of acetone and 0.2 ml. of 1% ammonium molybdate solution in 1:3 hydrochloric acid. Swirl and shake gently. After at least 10 minutes, read at either 430 m μ or 535 m μ . Calculate as follows:

$$\frac{A \times C \times \text{av. wt. of tablet in mg.}}{B \times \text{wt. of sample in aliquot in mg.}} = \text{mg. of iproniazid base per tablet}$$

in which *A* is the absorbance of the sample solution; *B* is the absorbance of the standard solution; and *C* is mg. of standard in the aliquot used.

Syrups. Prepare the sample and standard solution as described under tablets. To 20 ml. of the standard solution, add 20 ml. of the sample solution and dilute to 50 ml. with water. Calculate the concentration of the standard added as mg. of iproniazid base per ml. This is solution A.

Dilute 20 ml. of sample solution to 50 ml. with water and calculate the sample concentration as ml. of syrup per ml. This is solution B. Prepare three small flasks with narrow necks, one containing 1 ml. of water as a blank, one containing 1 ml. of solution A, and one containing 1 ml. of solution B. Into each, pipet exactly 10 ml. of acetone, and 0.2 ml. of 1% ammonium molybdate solution in 1:3 hydrochloric acid. Swirl and shake gently. Let stand for at least 10 minutes. Read solutions A and B at 430 $m\mu$ and calculate as follows:

$$\frac{B \times C}{(A - B) \times D} = \text{mg. of iproniazid base per ml. of syrup}$$

in which *A* is the absorbance of solution A; *B* is the absorbance of solution B; *C* is the mg. of standard from solution A in aliquot used; and *D* is mg. of sample.

NIALAMIDE, *N*-BENZYL- β -(ISONICOTINYLDIAZINO) PROPIONAMIDE

For nialamide and isopronazid by sodium nitritopentacyanoferroate, see page 995.

PYRAZINAMIDE, PYRAZINE CARBOXAMIDE

Pyrazinamide and cycloserine can be determined simultaneously in blood samples by the red color formed by reaction with sodium nitritopentacyanoferroate.¹⁶¹ There is a partial loss of cycloserine resulting from coprecipitation with serum proteins and also from destruction by the tungstic acid precipitating reagent. To compensate for this, calculate cycloserine values from a standard curve prepared with standard solutions of cycloserine dissolved in blood serum. High blank values result in serum samples containing a high value of nonprotein nitrogen. Nicotinic acid, isonicotinic acid, and thiazetazone interfere. At high concentrations, *p*-aminosalicylic acid interferes. Streptomycin and kanamycin may be present. Beer's law is followed for 2.5-10 mg. per 100-ml. sample. At 10°, the reaction products are stable for 20-60 minutes.

After hydrolysis by potassium hydroxide to pyrazinoic acid, pyrazinamide forms a yellow color when treated with hydrogen peroxide¹⁶² (cf. Vol. IV, pp. 261-2). There is no interference from pyrazine monocarboxylic acid, inorganic salts other than heavy metals and ammonium

¹⁶¹ K. B. Bjornesjo, *Scand. J. Clin. Lab. Invest.* **13**, 332-6 (1961).

¹⁶² Dai Aoki, Yoji Iwayama and Ikuko Furusumi, *Yakuzai-gaku* **16**, 9-11 (1956).

salts, nicotinic acid, and its amide, acetamide, hydrazine, isnicotinoyl monoethanolamide, and sodium *p*-aminosalicylate. Isonicotinic acid hydrazide, sodium isonicotinoyl methanesulfonate, some oximes and some components of body liquids also react with the reagent, and therefore, this method cannot be used in the analysis of blood or urine samples. Pyrazinamide concentration may be determined by reading in the ultraviolet at 269 $m\mu$.¹⁶³

Sample—Serum. To prepare the tungstic acid reagent, mix equal parts of 10% sodium tungstate solution and 1:52 sulfuric acid. Mix a 2-ml. sample with 4 ml. of tungstic acid reagent. Centrifuge at 2500 rpm for 10 minutes to remove serum proteins. Develop with sodium nitritopentacyanoferroate.

Procedure—By sodium nitritopentacyanoferroate. *Pyrazinamide and cycloserine simultaneously.* To prepare the reagent, mix equal parts of 4% sodium nitroprusside solution and 16% sodium hydroxide solution. To a 3-ml. protein-free sample, add 1 ml. of 18% acetic acid. Cool to 10° and add 1 ml. of cold sodium nitritopentacyanoferroate reagent. Maintain at 10° for 20 minutes and read at 490 and 625 $m\mu$ against a blank containing 3 ml. of water, 1 ml. of 18% acetic acid, and 1 ml. of reagent. The maximum for cycloserine is 625 $m\mu$, and that for pyrazinamide is 490 $m\mu$.

By hydrogen peroxide. Mix a 5-ml. sample containing up to 0.2 mg. of pyrazinamide per ml. with 0.5 ml. of 6.5% cobalt chloride hexahydrate solution and 2 ml. of 50% glycerol. Add 2 ml. of 11% potassium hydroxide with shaking and let stand for 5 minutes. Add 0.5 ml. of 3% hydrogen peroxide solution, dropwise, and let stand for 5 minutes. Filter, and read the yellow color within 1 hour.

In the ultraviolet. Read a blood sample deproteinized with trichloroacetic acid or a urine specimen that has been extracted with chloroform at 269 $m\mu$.

ETHIONAMIDE, 3-ETHYLISOTHIONICOTINAMIDE

The reaction of *p*-phenylenediamine and cyanogen bromide with ethionamide produces a purple color read at 460 $m\mu$.¹⁶⁴ 2-Ethylpyridine,

¹⁶³ Masatsugu Abe, *Kosankinbyo Kenkyu Zasshi* 12, 443 (1957).

¹⁶⁴ P. Kraus, *Ceskosl. farm.* 12, 246-9 (1963).

pyridine, isoniazid and its metabolites and derivatives, nicotinic acid, and nicotinamide give similar reactions with the reagents. Pyridoxal, pyridoxol, and pyridoxamine do not react. The accuracy of the method is $\pm 2.5\%$. Proteins must be separated.

In methanol, ethioniamide shows a maximum at 290 $m\mu$, in 1:110 hydrochloric acid, two maxima at 330 and 277 $m\mu$. At both points, Beer's law is followed up to 0.04 mg. per ml.¹⁶⁵ The recovery of ethioniamide by chloroform and hydrochloric acid extraction is 86%. By this technic, 0.0003-0.0004 mg. per ml. can be detected. Pyrazinamide interferes. Isoniazid, streptomycin, *p*-aminosalicylic acid, and cycloserine do not interfere.

Procedure—*By p-phenylenediamine and cyanogen bromide.* To 1 ml. of an approximately neutral protein-free solution of the sample containing 0.005-0.04 mg. of ethioniamide, add 1 ml. of an acetate buffer solution at pH 6. Add 0.5 ml. of 0.05% *p*-phenylenediamine solution and 0.5 ml. of 0.5% cyanogen bromide solution. After 9-16 minutes, read at 460 $m\mu$.

In the ultraviolet. Serum and other biological fluids. Extract ethioniamide into 30 ml. of chloroform. Reextract with 6 ml. of 1:110 hydrochloric acid and read at 315 $m\mu$.

PYRAZOLE, 1,2-DIAZOLE

This precursor of β -(pyrazolyl-*N*)-alanine is a product of microbiological degradation of the amino acid found in melon seeds. Since it contains the *N-N* linkage, it forms a characteristic complex applicable to hydrazines with pentacyanoaminoferroate.¹⁶⁶ The same reaction is applicable to isoniazid and unsymmetrical dimethylhydrazine. The color is stable for several hours. Heating does not increase the absorbance.

Stable colors are produced by this reaction with indazole, 3-amino-4-cyanopyrazole, 3-methyl pyrazole, 4-bromo-3-methyl pyrazole, 4-bromo pyrazole, 3,5-dimethyl pyrazole, and 4-bromo-3,5-dimethyl pyrazole. The same color is produced by *N*-hydroxymethyl pyrazole, 1-hydroxymethyl-3,5-dimethyl pyrazole, and 3,5-dimethyl pyrazole. In many cases, the hydroxymethyl group is probably split off. Colors that fade on addition of acid are formed with 4-amino-3,5-dimethyl pyrazole, 3,4-diamino-5-hydroxy pyrazole, and 4-amino pyrazole (3,4-*d*)pyrimidine. There is no color with 1-phenyl-3-methyl pyrazole or β (pyrazolyl-*N*)alanine.

¹⁶⁵ A. Bieder, D. Gerbail and L. Mazeau, *Ann. Pharm. Franc.* 19, 200-11 (1961).

¹⁶⁶ L. A. Larue, *Anal. Chem.* 37, 246-8 (1965).

Procedure—*Bacterial media.* Centrifuge to remove cells. To a sample containing 0.07-0.7 mg., per ml., add 1 ml. of 0.2% trisodium pentacyanoaminoferroate solution. Add 0.1 ml. of fresh 10% sodium nitrite solution. Mix, and at once add 0.1 ml. of glacial acetic acid. Read at 485 m μ .

PYRIDINE CHLOROPHENIRAMINE MALEATE,
2-[*p*-CHLORO- α -(2-DIMETHYLAMINOETHYL) BENZYL]

Chloropheniramine maleate is coupled with *p*-anisidine, the colored complex extracted into chloroform, and read at 487 m μ .¹⁶⁷ The color with sodium nitroprusside in sodium carbonate solution¹⁶⁸ and that with aniline and cyanogen bromide¹⁶⁹ have also been applied quantitatively. A variant uses sulfanilic acid.¹⁷⁰ Methapyrilene, mepyramine, norephedrine, phenylephrine, racephedrine, and dextromethorphan salts do not interfere.

Another technic is to steam distil the sample.¹⁷¹ An appropriate aliquot is passed through a column of Amberlite CG-50 in the hydrogen form. After elution with a buffer for pH 2, it is read directly at 264.5 m μ .

Chlorpheniramine maleate can be determined by the method for quaternary ammonium compounds (p. 192) using bromothymol blue and ethylenedichloride, even when aminopyrine is present.¹⁷² In the presence of ephedrine, either bromothymol blue or bromocresol purple can be used with benzene.

Sample—*Tablets.* Extract a sample containing up to 20 mg. of chloropheniramine maleate with an acetate buffer for pH 5 and then with three 5-ml. portions of water. Add 2.5 grams of sodium chloride to the combined extracts. Extract with 35, 25, 10, and 10 ml. of chloroform. Evaporate the combined extracts to dryness. Dissolve the residue in water and dilute to 100 ml.

Cough syrup. Such a sample may contain codeine, ephedrine, vasaka extract, camphor, menthol, and essential oils. Wash an acidified aqueous

¹⁶⁷ Kazuo Sato and Mitsuaki Takada, *Arch. Pract. Pharm., Japan* **22**, 57-9 (1962).

¹⁶⁸ Kiichiro Kakemi, Toyozo Uno and Hitoshi Sezaki, *J. Pharm. Soc. Japan* **76**, 273-6 (1956).

¹⁶⁹ D. J. Vadodaria, P. M. Parikh and S. P. Mukherji, *Indian J. Pharm.* **24**, 180-3 (1962).

¹⁷⁰ J. Hudanick, *J. Pharm. Sci.* **53**, 332-3 (1964).

¹⁷¹ Tetsuyasu Hattori and Michinori Nichiumi, *Arch. Prac. Pharm., Japan* **21**, 9-14 (1961).

¹⁷² Masayoshi Horioka and Hiromi Ishioka *Yakugaku Zasshi* **81**, 76-9 (1961).

solution with chloroform and discard the washings. Make the aqueous phase alkaline with ammonia and extract the chloropheniramine with chloroform. Evaporate the chloroform to dryness, take up the residue in 1:100 hydrochloric acid, and neutralize.

Procedure—*Cyanogen bromide and p-anisidine*. To a 5-ml. aliquot, add 3 ml. of acetate buffer for pH 6 and 2 ml. of 2% cyanogen bromide solution. Let stand at room temperature for 15 minutes. Add 1 gram of sodium chloride and 5 ml. of 0.5% *p*-anisidine solution and shake vigorously for 10 seconds. Let stand for 30 minutes in the dark and discard the upper layer. Read the organic layer at 487 $m\mu$.

Cyanogen bromide and sulfanilic acid. As a reagent, dissolve 2.5 grams of sulfanilic acid and 4 grams of anhydrous sodium acetate in 40 ml. of water and dilute to 175 ml. with 95% ethanol. To a 1-ml. sample containing about 0.04 mg. of chloropheniramine, add 7 ml. of the reagent and 3 ml. of 4% aqueous cyanogen bromide. Read at 480 $m\mu$ in 10 minutes against a reagent blank.

PIPERAZINE, DIETHYLENEDIAMINE

When reacted with an alcoholic solution of *p*-benzoquinone, piperazine forms a red-orange colored complex with a maximum absorbance at 490 $m\mu$. It follows Beer's law in the range of 0.03-0.2 mg. per ml.

Phenothiazine, dibutyltin, sulfaquinoxaline, and furazolidone do not interfere. When the piperazine is read at 490 $m\mu$, the temperature must be controlled at 80° and the heating period of 10 minutes must be exact. A long heating period results in a more stable color with a maximum at 500 $m\mu$.¹⁷³

By reaction with acetic anhydride in sodium bicarbonate solution, piperazine forms diacetyl piperazine, which is extracted with chloroform and read in the infrared.¹⁷⁴ A standard of diacetyl piperazine is prepared from the diacetyl piperazine recovered from the acetylation of a piperazine dihydrochloride standard. The material is recrystallized twice from benzene. Beer's law is followed for 0.02-0.2 gram of diacetyl piperazine.

¹⁷³ Sam H. Perlmutter, *J. Assoc. Offic. Agr. Chemists* **41**, 306-8 (1958); *ibid.* **42**, 463-4 (1959); Herman F. Beckman and Lutrell Feldman, *J. Agr. Food Chem.* **8**, 227-8 (1960).

¹⁷⁴ Wm. R. Maynard, Jr., *J. Assoc. Offic. Agr. Chemists* **42**, 610-12 (1959).

Piperazine may also be developed by Nessler's reagent.¹⁷⁵

For determination of piperazine in the presence of hexamine, it is precipitated as the reineckate.¹⁷⁶ This reaction in alkaline solution is in a 1:1 ratio; in acid solution, in a 1:2 ratio. It is read in citric acid solution of pH 2 at 525 m μ . If other quaternary ammonium compounds that form reineckates in alkaline solution are not present, it is read at pH 9.2.¹⁷⁷

There are various methods of isolating the piperazine from the sample. It may be separated by steam distillation from an alkaline extract of the sample.¹⁷⁸ Interferences may be extracted from an acidic sample by passage through Hyflo Supercel.¹⁷⁹ Then the solution is made alkaline prior to color development. By dissolving the sample in 4% sodium hydroxide solution, the piperazine is liberated. It is extracted with chloroform and reacted with erythrosine.¹⁸⁰

Sample—Powdered products. Mix by rolling or shaking. Take a sample that represents 50-100 mg. of piperazine and dilute to 1 liter with water. Add the sample solution to the flask for steam distillation. Add 5 grams of sodium chloride and 5 ml. of 50% sodium hydroxide. Heat to boiling and distil 200 ml. Develop a 20-ml. portion of the distillate with 1,4-benzoquinone.

Syrups. Dilute a 50-ml. sample with water so that 10 ml. represents 50-100 mg. of piperazine. Follow the procedure under "Powdered products."

Feeds. Separation by steam distillation. The steam distillation apparatus consists of the following: Steam is generated from water in a 1-liter flask containing the necessary fittings and is delivered to the bottom of a 300 ml. Kjeldahl flask, which contains the feed extract, through a glass tube 6-mm. in outside diameter. This tube is passed through one hole of the 2-hole stopper that closes the Kjeldahl flask. The inlet of the Kjeldahl bulb, a type that does not retain water, is passed through the other hole. The exit tube from the Kjeldahl bulb is bent and

¹⁷⁵ R. P. Chakravarti and N. K. Dey, *J. Proc. Inst. Chemists (India)* **31**, 53-4 (1959).

¹⁷⁶ M. Masse, *Pharm. Acta Helv.* **33**, 80-4 (1958).

¹⁷⁷ R. E. Pankratz, *J. Pharm. Sci.* **50**, 175-7 (1961).

¹⁷⁸ J. W. Cavett, *J. Assoc. Offic. Agr. Chem.* **41**, 323-5 (1958).

¹⁷⁹ Herman F. Beckman and Laurell Feldman, *J. Agr. Food Chem.* **8**, 227-8 (1960)

¹⁸⁰ C. Pfeiffer and M. Hasselmann, *Chim. anal.* **43**, 321-4 (1961).

connected to a 20-cm. bulb condenser held in a vertical position. A 25-cm. glass delivery tube, 10 mm. in outside diameter, is connected to the condenser with a rubber tube. The open end of the delivery tube extends to the bottom of a 200-ml. volumetric flask, and as the distillation proceeds, the tube is kept above the surface of the distillate.

Extract feeds, containing 0.055% to 0.4% of piperazine, with water measured so that each 20 ml. of extract will contain 2-4 mg. of piperazine. For example, for feeds containing 0.2% of piperazine, mix 10 grams of sample with 200 ml. of water and adjust the pH to 5 with 1:2 sulfuric acid. Let stand for 30 minutes, shaking frequently, or centrifuge a portion of the extract at 2000 rpm for 5 minutes. Pipet a 20-ml. aliquot of the extract into the 300-ml. Kjeldahl flask. Add 1 drop of Antifoam G-60, 10 grams of sodium chloride, and 10 ml. of 50% sodium hydroxide solution. Immediately connect the flask to the steam distillation apparatus and collect the distillate. Develop with 1,4-benzoquinone.

Feeds. Extraction of piperazine by passage through Hyflo Supercel. Prepare a standard solution by diluting 200 mg. of piperazine to 100 ml. with water and mix well. Weigh a 10-gram sample into each of six 250-ml. flasks. Add 0, 5, 10, 15 and 20 ml. aliquots of the standard piperazine solution to the flasks. Add 0.3 ml. of 1:2 sulfuric acid to each and dilute each to 100 ml. Shake intermittently for 30 minutes. Allow the heavy particles to settle for approximately 10-15 minutes. Add filter paper to six Büchner funnels and cover the paper with a one-quarter-inch layer of Hyflo Supercel, spreading evenly. Apply suction. Wet the entire surface of the Supercel with a few ml. of the extract. Release the suction and discard the filtrates. Reconnect the flasks and suction and filter the remaining extracts. If the filtrate is not clear, refilter. Dilute a 20 ml. portion of each filtrate to 200 ml. with water. Develop an aliquot of each with 1,4-benzoquinone.

Procedure—By 1,4-benzoquinone. To purify 1,4-benzoquinone, steam distil 12-gram batches into a collecting flask immersed in cold water. Collect the crystals on a glass filter, air dry, and grind. Dissolve 0.5 gram of purified quinone in 2.5 ml. of glacial acetic acid and dilute to 100 ml. with ethanol. Refrigerate, and prepare fresh after 48 hours.

Dilute a 20-ml. portion of the sample to 500 ml. with water. To a 5-ml. aliquot, add 5 ml. of quinone reagent. Mix by inverting and heat at 80°, or slightly higher, for 30 minutes. Cool for 1 minute in water and

dilute to 10 ml. with ethanol. Mix by inverting and let stand for 45 minutes. Read against a reagent blank at 500 $m\mu$.

By acetylation, infrared. Solutions or syrups. Dilute a sample equivalent to 0.05 gram of piperazine base to 25 ml. with water. Neutralize with 4.5 grams of sodium bicarbonate and shake. Add 1 ml. of acetic anhydride and swirl slowly to release carbon dioxide. After evolution of carbon dioxide stops, add another 1 ml. of acetic anhydride and swirl again. Add additional sodium bicarbonate if necessary to maintain a slight excess. After the carbon dioxide evolution ceases, let the sample stand at room temperature for 30 minutes. Extract with one 25-ml. portion and five 15-ml. portions of chloroform, filtering each portion through cotton. Evaporate the chloroform to dryness at 100° with the aid of a stream of air. Take up the residue with 5 ml. of chloroform and transfer to a 10-ml. flask. Rinse the beaker with enough chloroform to increase the volume in the flask to 10 ml. Compare the absorbance of the sample with that of the standard at 10.03 μ with a fixed slit opening at 220.

Tablets and wafers. Weigh a total of 20 tablets or 10 wafers and obtain an average weight per tablet or wafer. Grind, and weigh a portion equivalent to 0.5 gram of piperazine base. Dilute to 100 ml. with water and mix for 15 minutes. Filter, discarding the first 10 ml. of the filtrate. Dilute a 10-ml. aliquot to 20 ml. with water, and follow the procedure for solutions and syrups, starting at "Neutralize with 4.5 grams of sodium bicarbonate and shake. . . ."

By Nessler's reagent. Dilute a sample to contain 0.5 gram of piperazine hydrate or its salts per 100 ml. To a 5-ml. aliquot, add 2 ml. of Nessler's reagent and dilute to 50 ml. Compare the turbidity with standards.

HYDROXYZINE, 1-(*p*-CHLORO- α -PHENYLBENZYL)-
4-(2-HYDROXYETHOXY)-ETHYL PIPERAZINE

Hydroxyzine can be developed nephelometrically with sodium tetraphenylboron or read directly in the ultraviolet.¹⁸¹

Procedure—Fccds. Blend a sample containing 0.1-0.4 mg. of hydroxyzine hydrochloride with 250 ml. of chloroform for about 3 minutes. Filter

¹⁸¹ L. P. Tuttle and E. D. Schall, *J. Assoc. Offic. Agr. Chemists* **47**, 228-31 (1964).

on a Büchner funnel and wash with 50 ml. of chloroform. Evaporate to about 75 ml. with aspiration.

Extract with 20, 20, and 20 ml. of 1:20 hydrochloric acid. Wash the combined extracts with 10 and 10 ml. of isooctane, discarding the washings. Add 12% sodium hydroxide solution dropwise to the combined acid extracts until the pH is 10.5-11.0. Extract with 20, 20, and 20 ml. of chloroform. Wash the combined chloroform extracts with 5 ml. of water. Dry the chloroform extract by filtering through 5 grams of anhydrous sodium sulfate and wash with additional chloroform. Evaporate the chloroform extract to 10-15 ml. by aspiration and gentle heat.

Nephelometric. Transfer to a separatory funnel, using additional chloroform for washing. Add 1.5 volumes of isooctane. Extract with 5 ml. and 5 ml. of 1:100 hydrochloric acid by shaking gently for about 3 minutes with each.

As a reagent, dissolve 20 grams of sodium tetraphenylborate in about 800 ml. of water. Add 20-25 grams of aluminum hydroxide and stir for 10 minutes. Filter, collecting the cloudy forerun separately for refiltering. Add 2 ml. of 20% sodium hydroxide solution and dilute to 1 liter with water. For use, dilute 1:10.

As a buffer dissolve 60 grams of glacial acetic acid in about 800 ml. of water. Add solid sodium hydroxide to adjust the pH to 5.2 and dilute to 1 liter with water. Add 5 ml. of the acid extract of the sample to a nephelometer tube with 8 ml. of buffer. After mixing, add 2 ml. of the tetraphenylborate reagent with constant agitation. Read nephelometrically.

Spectrophotometric. Shake 20 grams of aluminum oxide with 1 ml. of water until the texture is uniform. Slurry with chloroform and transfer to a 7.5×150 mm. chromatographic column. Pack by tapping and drain the chloroform to 1 cm. above the bed. Prepare a fresh column for each determination.

Transfer the chloroform solution of the sample to the column and wash in with minimal amounts of chloroform. Add additional chloroform as necessary and collect 75 ml. of effluent. Reduce this to 10-15 ml. by warming. Transfer to a separatory funnel and wash in with 5 ml. of chloroform. Add 1.5 volumes of isooctane and extract the test substance with 5 ml. and 5 ml. of 1:100 hydrochloric acid, gently shaking about 3 minutes with each. Read the absorbance of the combined extracts at $232\text{ m}\mu$ against 1:100 hydrochloric acid.

5-AMINO-1,2-DIMETHYLIMIDAZOLE

When diazotized and coupled with *N*-1-naphthylethylenediamine, 5-amino-1,2-dimethylimidazole gives a stable red-orange color with an absorption maximum at 495 $m\mu$.¹⁸² 1,2-Dimethyl-5-nitroimidazole may be determined by this method after reduction to 5-amino-1,2-dimethylimidazole. The method is applicable to 0.005-0.03 mg. per ml.

Procedure—Homogenize the sample and deproteinize with trifluoroacetic acid. Centrifuge and dilute a portion of the supernatant liquid containing 0.005-0.03 mg. to 2 ml. with 2:3 hydrochloric acid. Add 1 ml. of 1% sodium nitrite solution and let stand for 10 minutes. Remove excess nitrite with 2 ml. of 2% ammonium sulfamate solution. When all sodium nitrite is decomposed, add 1 ml. of 1% *N*-1-naphthylethylenediamine solution and read at 495 $m\mu$ against a reagent blank.

INDOXYL

A reagent for indoxyl is ferric chloride in hydrochloric acid¹⁸³ (Vol. IV, pp. 287-8).

Procedure—*Serum or plasma*. Deproteinize a 5-ml. sample by adding 10 ml. of 20% trichloroacetic acid solution and 5 ml. of water. Filter or centrifuge. To 15 ml. of the filtrate or supernatant liquid, add 10 drops of 5% thymol solution in alcohol and 15 ml. of 0.5% ferric chloride in solution in concentrated hydrochloric acid. Incubate for 30 minutes at 40° and shake with 5 ml. of chloroform. Read the chloroform extract at 530 $m\mu$.

3-AMINO-1H-1,2,4-TRIAZOLE

Aminotriazole in trichloroacetic acid solution is diazotized with nitrous acid, and the diazo compound formed is coupled with chromotropic acid, 4,5-dihydroxynaphthalene-2,7-disulfonic acid, to form a pink derivative.¹⁸⁴ Chromotropic acid itself produces a light yellow color. Therefore, the pink color is read at 525 $m\mu$, at which the chromotropic acid blank

¹⁸² L. B. Colvin, R. Silvaramakrishnan and J. R. Couch, *Chemist Analyst* 52, 9-11 (1963).

¹⁸³ Virginia Ballarte Romero, *Cron. Med.* 68, 137-48 (1951).

¹⁸⁴ Frank O. Green and Robert N. Feinstein, *Anal. Chem.* 29, 1658-60 (1957).

color does not affect the readings. The procedure is applicable to 0.01-0.125 mg. of the triazole. However, Beer's law is not followed. Trichloroacetic acid serves as the deproteinizing agent.

The following compounds also give a color with the reagent: 3-hydroxy-1,2,4-triazole, 1-amino-2-naphthol-4-sulfonic acid, 2-aminopyridine, 2-aminopyrimidine, cysteine, dihydroxyphenylalanine, guanine, histamine, histidine, 1-naphthylamine, phenylalanine, tryptophan and tyrosine. The formation of a yellow color in the chromotropic acid reagent can be minimized by addition of a small amount of ascorbic acid or other stabilizer.

The same reaction is applicable with 8-amino-1-naphthol-3,6-disulfonic acid (H acid).¹⁸⁵ The color developed with *N*-1-naphthylethylenediamine dihydrochloride is read at 455 m μ .¹⁸⁶ The sensitivity is to 0.025 ppm.

Procedure—Mix an aqueous sample containing up to 0.12 mg. of 3-amino-1,2,4-triazole with an equal volume of 10% trichloroacetic acid solution, and filter, if needed. If it is necessary to bring the aminotriazole concentration to the desired range, dilute with 5% trichloroacetic acid. To a 5-ml. aliquot, add 1 ml. of 0.069% sodium nitrite solution and mix by swirling. Add 1 ml. of freshly prepared 0.089% chromotropic acid solution and heat at 100° for 2.5 minutes. Cool in ice and read at 525 m μ against a 5% solution of trichloroacetic acid solution that has been carried through the procedure.

ANTIPYRINE, 1,5-DIMETHYL-2-PHENYL-3-PYRAZOLONE

An aliquot of protein-free filtrate containing antipyrine is nitrosated in glacial acetic acid with sodium nitrite, and the resulting nitroso compound is condensed with 1-naphthylamine to form an azo dye.¹⁸⁷ With a concentration of 15% sodium nitrite, the color formed is purple with a maximum at 580 m μ . With lower concentrations of sodium nitrite, the maximum is at 540 m μ . The intensity of the color is related to the period of nitrosation. The color is stable for 1 hour and fades slightly over a period of 24 hours. Sulfonamides and *p*-aminohippuric acid interfere.

¹⁸⁵ R. A. Herrett and A. J. Linck, *J. Agr. Food Chem.* **9**, 466-7 (1961).

¹⁸⁶ R. W. Storherr and J. Burke, *J. Assoc. Offic. Agr. Chemists* **44**, 196-9 (1961).

¹⁸⁷ D. Mendelsohn and N. W. Levin, *J. Lab. Clin. Med.* **54**, 311-18 (1959); Cf. Yahyoe Kinoshita and Shigetaka Moriyama, *Bull. Nagoya City Univ. Pharm. School* **No. 2**, 28-30 (1954).

Pethidine, aspirin, aminopyrine, and phenobarbitone do not interfere. Beer's law is followed up to 0.05 mg. of antipyrine per ml. Condensation with *N*-1-naphthylethylenediamine is an alternative with reading at 592 m μ .¹⁸⁸

The method for antipyrine by diazotizing and coupling with *N*-1-naphthylethylenediamine is also applicable to 4-aminoantipyrine. Acetylation of 4-aminoantipyrine avoids its interference with determination of antipyrine by diazotized nitroaniline (Vol. IV, p. 291).¹⁸⁹

Antipyrine is precipitated by molybdophosphate. The excess reagent is masked by tartrate and the sexivalent molybdenum in the precipitate is reduced to molybdenum blue, which is read at 750 m μ .¹⁹⁰ The same reaction is given by pilocarpine, caffeine, and morphine. There is no interference by aminopyrine, quinine, atropine, aniline, and ephedrine.

The OH group in the antipyrine molecule condenses with *p*-dimethylaminobenzaldehyde in a hydrochloric acid medium to form a colored product, which follows Beer's law (Vol. IV, pp. 281-2). Because of the reaction of the OH group, it is possible to determine antipyrine in the presence of aminopyrine.¹⁹¹ In the reaction of antipyrine with *p*-dimethylaminobenzaldehyde, aspirin, phenacetin, antifebrin, and pyramidon do not interfere. The determination can be performed with as little as 0.03 mg. per ml.¹⁹² Ferric nitrate is also a reagent for antipyrine.¹⁹³ Aromatic amines, quinones, phenacetin, codeine, and sugars do not interfere. Any substances that react with ferric ion do interfere.

Procedure—By sodium nitrite and 1-naphthylamine. *Plasma*. To prepare the cadmium reagent, dissolve 104 grams of cadmium sulfate in water. Add 508 ml. of 1:35 sulfuric acid and dilute to 1 liter with water.

To a 2-ml. sample, add 1 ml. of the cadmium reagent with mixing. Add 1 ml. of 4.4% sodium hydroxide solution and stir well. Centrifuge for 5 minutes at 3000 rpm and for 3-4 minutes at 1500 rpm.

Prepare the following glass-stoppered tubes: (1) Blank—1.5 ml. of water; (2) Standard—1 ml. of solution containing 0.02 mg. of antipyrine in water plus 0.5 ml. of water; (3) Test—1.5 ml. of protein-free filtrate.

Add 5 ml. of glacial acetic acid and 0.25 ml. of 15% sodium nitrite

¹⁸⁸ D. Mendelsohn and N. W. Levin, *J. Med. Sci.* **25**, 13-18 (1960).

¹⁸⁹ R. Hasslinger and W. Strunz, *Arzneimittel-Forsch.* **5**, 61-2 (1955).

¹⁹⁰ J. Celechovsky and D. Svobodova, *Ceskoslov farm.* **8**, 380-4 (1959).

¹⁹¹ Rozsa Pal, *Acta Pharm. Hung.* **27**, 246-56 (1957).

¹⁹² M. Hahn, J. Kolsek and M. Perpar, *Z. anal. Chem.* **151**, 104-8 (1956).

¹⁹³ Jaroslav Celechovsky and Vladimir Krejci, *Ceskoslov farm.* **6**, 98-103 (1957).

solution to each tube. Mix, and let stand for 20 minutes. Add 0.25 ml. of 75% ammonium sulfamate solution and shake vigorously for 3-5 minutes or until the evolution of gas stops. Add 1 ml. of freshly prepared 10% 1-naphthylamine solution in glacial acetic acid with mixing, and heat at 50° for 5 minutes. Cool in cold water and let stand at room temperature for 20 minutes. Read at 580 m μ .

By p-dimethylaminobenzaldehyde. As a reagent, dissolve 0.1 gram of *p*-dimethylaminobenzaldehyde in 4 ml. of 1:9 hydrochloric acid and dilute to 20 ml. To a solution containing 0.01-0.1 mg. of antipyrine, add 1 ml. of *p*-dimethylaminobenzaldehyde reagent. Stopper, and immerse to a 5-cm. depth in a 80-90° bath for 5 minutes. Let stand at room temperature for 30 minutes. Add 3 ml. of acetone and read.

As molybdenum blue. To prepare the stannous chloride reducing solution, dissolve 0.15 gram of tin in 3 ml. of concentrated hydrochloric acid. Add a few drops of 5% cupric sulfate solution and dilute to 100 ml. To a 5-ml. solution containing 1-5 mg. of antipyrine, add 5 ml. of a 6.33×10^{-3} M solution of molybdophosphate. Add 5 ml. of 15% tartaric acid solution. After 8 minutes, add 10 ml. of stannous chloride solution to reduce the molybdenum to molybdenum blue. Dilute to 50 ml. and read at 750 m μ .

By ferric nitrate. Adjust the sample solution containing 0.1-30 mg. of antipyrine to pH 2. Add 5 ml. of 17.5% ferric nitrate solution and read at 470 m μ , after dilution if necessary.

4-ACETAMIDOANTIPYRINE

When 4-acetamidoantipyrine is heated with potassium ferrieyanide and phenol, a pink color is developed, which is read at 517 m μ .¹⁹⁴ The limit of detection is 0.002 mg. per ml. Other phenolic bodies may also be used. The color and limit of detection in micrograms per ml. is as follows: phenol pink, 2; *o*-cresol pink, 4; *m*-cresol orange, 2; resorcinol orange, 2; phloroglucinol violet, 4; oxine red, 4; chromotropic acid violet, 8.

Sample—Plasma. Heat a 2-ml. sample with 1 ml. of 1:5 hydrochloric acid in a glass-stoppered tube at 100° for 30 minutes. Cool. Add 7 ml. of boric acid-sodium hydroxide buffer containing 2.47% of boric acid in

¹⁹⁴ Toshio Nambara and Takako Urakawa, *Yakugaku Zasshi* **80**, 1663-7 (1960).

0.12% sodium hydroxide solution. Add 0.3 ml. of 0.3% phenol solution and 0.2 ml. of 0.4% potassium ferricyanide solution. Read at 517 m μ against water.

NOVALGIN, METHYLAMINOANTIPYRINE, AND MELUBRIN, SULFAMIPYRINE

A pink color, which is the oxidation product, forms when methylaminoantipyrine is treated with iodine.¹⁹⁵ Novalgin and Melubrin also give a yellow color with dimethylaminobenzaldehyde.¹⁹⁶ The maximum absorption is at 420 m μ . The absorption of Melubrin is 5 times that of Novalgin, but only after hydrolysis to aminopyrine.

Procedure—*By iodic acid.* To a 3-ml. sample containing up to 0.1% of methylaminoantipyrine, add 2 drops of concentrated sulfuric acid and 2 ml. of 1% iodic acid solution. Heat for 30 minutes to 90-100°. Extract with 5 ml. of chloroform and compare the pink color formed with standards.

Alternatively, to 5 ml. of a similar sample, add 0.5 ml. of phosphomolybdic acid reagent (p. 322), and after 0.5-48 hours compare with standards.

By dimethylaminobenzaldehyde. Novalgin. To 1 ml. of sample containing about 0.4 mg. of Novalgin, add 4 ml. of methanol and 3 ml. of a buffer for pH 4. As reagent, dissolve 1 gram of *p*-dimethylaminobenzaldehyde in 100 ml. of 95% ethanol and 10 ml. of concentrated hydrochloric acid. Add 1 ml. of this reagent, and after 15 minutes read at 420 m μ .

Melubrin. This hydrolyzes in aqueous solution to formaldehyde and aminoantipyrine. The latter reacts with the reagent to absorb at 420 m μ .

2-HYDROXY-4,6-DIMETHYLPYRIMIDINE

In an alcoholic sulfuric acid solution, 2-hydroxy-4,6-dimethylpyrimidine forms a red color upon diazotizing with nitrous acid and reaction with sulfanilic acid.¹⁹⁷ The red complex shows absorption peaks at 450

¹⁹⁵ Juan A. Sanchez, Rosa C. D'Alessio de Carnevale Bonino, *Rev. asoc. bioquim. argentina* **18**, 363-71 (1953).

¹⁹⁶ Antal Végh, Gyorgy Szasz and Piroska Kertesz, *Acta Pharm. Hung.* **31**, 1-7, 49-54 (1961).

¹⁹⁷ C. R. Szalkowski and W. J. Mader, *Anal. Chem.* **27**, 1404-8 (1955).

and 540 $m\mu$. The peak at 540 $m\mu$ is more sensitive and more specific than that at 450 $m\mu$. Beer's law is followed at 540 $m\mu$ for up to 0.075 mg. per ml.

The color is specific for 2-hydroxy-4,6-dimethylpyrimidine except for the reaction of 2-amino and 2-mercapto-4,6-dimethylpyrimidine. The following compounds produce no color with the reagents under the conditions of the procedure: 2-amino-5-chloropyrimidine, 2-amino-5-methylpyrimidine, 2-amino-5-nitropyrimidine, 2,4-dihydroxy-5-methylpyrimidine, 2-hydroxypyrimidine, 2-methyl-4-amino-5-ethoxymethylpyrimidine, 2-methyl-4-amino-5-bromomethylpyrimidine, 2-methyl-4-amino-5-nitratomethylpyrimidine, 2-methyl-4-amino-5-chloromethylpyrimidine, 2-methyl-4-hydroxy-6-ethoxymethylpyrimidine, pyrimidine, allantoin, alloxan, arsanilic acid, biuret, barbital, caffeine, cyanuric acid, hexamine, histamine, hydroxyproline, 2-hydroxypyridine, 4,4'-dinitrocarbanilide, niacin, niacinamide, nitrofurazone, pyridine, pyridoxine, phenobarbital, proline, theobromine, thiamine, uric acid, and guanine.

The following compounds produce a yellow color with the reagents: 2-amino-4-methylpyrimidine, 2-hydroxy-4-methylpyrimidine, aminopyrine, aniline, dinitrodiphenyl disulfide, *p*-aminosalicylic acid, *p*-nitroaniline, *p*-aminobenzoic acid, sulfanilamide, sulfathiazole, sulfamerazine, sulfadiazine, sulfapyridine, sulfamethazine, and 4-hydroxy-3-nitrophenylarsonic acid.

Procedure—Add 15 ml. of an ethanolic solution of the sample containing 0.002-0.004 mg. of 2-hydroxy-4,6-dimethylpyrimidine per ml. to 5 ml. of 1:119 sulfuric acid containing 20 mg. of sulfanilic acid per ml. Mix. Add 1 ml. of 6% sodium nitrite solution and dilute to 25 ml. with ethanol. Heat for 15 minutes at 65° and let stand at room temperature for 1 hour. Centrifuge the red turbid liquid and read the supernatant layer at 540 $m\mu$.

OROTIC ACID, 1,2,3,6-TETRAHYDRO-2,6-DIOXO-4-PYRIMIDINECARBOXYLIC ACID

After treatment with bromine, orotic acid is developed with *p*-dimethylaminobenzaldehyde.¹⁹⁸ The presence of 5 moles of the following substances per mole of orotic acid does not interfere: L-dihydroorotic acid, uracil, thymine, cytosine, alloxan, alloxantin, uric acid, iridine, guanine.

¹⁹⁸ Kazuyuki Tsuji, *Yakugaku Zasshi* **81**, 1655-6 (1961); Toru Adachi, *Bitamin* **27**, 433-7 (1963).

adenine, adenosine triphosphate, DL-citrulline, hydantoin, allantoin, ascorbic acid, thiamine, riboflavine, pyridoxine, nicotinic acid, nicotinamide, D-pantothenic acid, folic acid, DL-thioctic acid, carnitine, choline, glucuronic acid, glucosamine, N-acetylglucosamine, L-histidine, L-lysine, L-methionine, L-cysteine, L-arginine, L-asparagine and L-aspartic acid.

An alternative is to measure the decrease in absorbance at 295 m μ when orotic acid is converted to uridylic acid by yeast orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase.¹⁹⁹ This method is sensitive to one microgram.

Procedure—To a 1-ml. sample containing 0.001-0.015 mg. of orotic acid at pH 2-3, add 2 ml. of 5% solution of monopotassium citrate as a buffer for pH 2.5. Add 0.5 ml. of saturated aqueous bromine solution. After 10 seconds, add 0.5 ml. of 0.7% sodium mercaptoacetate solution to decolorize excess bromine. Heat at 56° for 3 minutes. Add 2 ml. of 2.5% *p*-dimethylaminobenzaldehyde solution in propanol and read at 480 m μ within 30 minutes against water.

In the presence of substances absorbing at 480 m μ , such as riboflavine, extract the solution with 4 ml. of butyl acetate and read the organic layer at 461 m μ .

2-METHYL-4-AMINO-5-AMINOMETHYLPYRIMIDINE

Ninhydrin in pyridine is a reagent for 2-methyl-4-amino-5-amino-methylpyrimidine.²⁰⁰ The 5-methylacetamide analog of the sample, sodium acetate, and sodium chloride do not interfere. Ammonium ion should be removed *in vacuo* as ammonia from alkaline solution.

Procedure—To a 1-ml. sample containing 0.05-0.3 mg. of 2-methyl-4-amino-5-aminomethylpyrimidine in a buffer for pH 4, add 1 ml. of 50% pyridine solution and 1 ml. of 1% ninhydrin solution. Heat for 30 minutes at 100°. Cool, and dilute to 25 ml. with water. Read at 570 m μ .

URACIL, 2,4-DIOXOPYRIMIDINE

For uracil, see purines, page 560.

¹⁹⁹ F. M. Rosenbloom and J. E. Seegmiller, *J. Lab. Clin. Med.* **63**, 492-500 (1964).

²⁰⁰ Satoshi Mizukami, Tameyuki Amano and Shigeko Sakamoto, *J. Pharm. Soc. Japan* **76**, 1082-4 (1956).

CYTOSINE, 4-AMINO-2(1)-PYRAMIDONE

For cytosine, see purines, page 560.

THYMINE, 5-METHYLURACIL

For thymine see purines, page 560.

2-THIOURACIL, 6-METHYL-2-THIOURACIL AND
6-PROPYL-2-THIOURACIL

Thiouracils give a green color with a reagent containing sodium nitroprusside and hydroxyammonium chloride.²⁰¹ Up to 200 ppm. may be measured. An alternative technic uses ammonia and potassium iodate for determining the 6-methyl and 6-propyl derivatives.²⁰² Lactose or starch do not interfere.

Procedure—*By sodium nitroprusside. Animal feedstuffs.* To prepare the buffer for pH 8 solution, dissolve 5.74 grams of sodium barbitone and 2.08 grams of glycine in 450 ml. of water. Add 11.7 ml. of 1:10 hydrochloric acid and dilute to 500 ml. Store at 5°. To prepare the reagent, dissolve 0.5 gram of sodium nitroprusside and 0.5 gram of hydroxyammonium chloride in 10 ml. of water. Add 1 gram of sodium bicarbonate and keep at 20°. Add 0.1 ml. of bromine, dropwise. Warm at 37° for 20 minutes and dilute to 25 ml. Store at 5°. Before use, dilute 1:10 with the barbitone buffer at pH 8 and maintain at 37° until the mixture is yellow.

Grind a 1.2-gram sample for 25 minutes with 200 ml. of water and 5 ml. of 5% sodium hydroxide solution. Dilute to 1 liter. Filter, and analyze immediately. To each of two 3.5-ml. aliquots, add 0.35 ml. of 0.33% acetic acid. To each of two 1-ml. aliquots, add 0.1 ml. of 0.33% acetic acid solution. Dilute all to 4 ml. with water. To one of the original 3.5-ml. aliquots and one 1-ml. aliquot, add 2 ml. of reagent. Read within 10 to 25 minutes at 690 m μ against a blank of 2 ml. of reagent and 4 ml. of water.

To the remaining aliquots, add 2 ml. of barbitone buffer at pH 8 and read at 690 m μ against the barbitone buffer. Refer the difference between

²⁰¹ F. Bucci and A. M. Cusmano, *R. C. 1st Sup. Sanit.* 25, 518-28 (1962).

²⁰² J. Bruno, *Boll. Chim. Farm.* 102, 478-80 (1963).

the extinction to standard curves containing 0.06-0.18 mg. of 2-thiouracil, 6-propyl-2-thiouracil, or 6-methyl-2-thiouracil. If the sample contains a mixture, use the curve for 6-methyl-2-thiouracil.

By potassium iodate. 6-Methyl-2-thiouracil. Tablets. Grind tablets containing about 100 mg. of test substance and add to 80 ml. of water. Add 0.8 ml. of concentrated ammonium hydroxide, shake, and filter. Mix 0.5 ml. of this solution, 5 ml. of glacial acetic acid, and 2 ml. of 0.5% potassium iodate solution. Dilute to 8 ml. with water. After 80 minutes, read at 465 $m\mu$.

6-Propyl-2-thiouracil. Grind tablets containing 50 mg. of test substance in 10 ml. of 95% ethanol. Add 30 ml. of water and let stand for 30 minutes. Filter, and develop as for the 6-methyl derivative.

2,4-DIOXO-3,3-DIETHYL-5-METHYLPIPERIDINE

2,4-Dioxo-3,3-diethyl-5-methylpiperidine is coupled with diazotized sulfamic acid, and the yellow color formed is read in isopropanol at 378 $m\mu$.²⁰³ Beer's law is followed for 0.001-0.05 mg. per ml. Another reagent for 2,4-dioxo-3,3-diethyl-5-methylpiperidine is the protein reagent of Folin and Ciocalteu.²⁰⁴ Beer's law is followed over the range 0.05-0.17 mg. per ml.

Procedure—*By diazotized sulfamic acid.* Diazotize 1 ml. of 1% sulfamic acid solution in 1:10 hydrochloric acid. Mix 5 ml. of a sample containing 0.001-0.05 mg. of 2,4-dioxo-3,3-diethyl-5-methylpiperidine per ml. with 2.5 ml. of 2% sodium hydroxide solution. Add the diazo reagent. Shake and warm at 30° for 10 minutes. Shake vigorously with 1.5 ml. of isopropanol and read the yellow color at 378 $m\mu$.

By Folin-Ciocalteu reagent. Treat a 1-ml. aqueous sample containing 0.052-0.170 mg. of 3,3-diethyl-2,4-dioxo-5-methylpiperidine with 1 ml. of a 1:2.5 Folin-Ciocalteu reagent (Vol. 3, p. 116) and water. Add 1 ml. of 4% sodium hydroxide solution and read at 600 $m\mu$.

²⁰³ Masaru Nakano, *Yakuzaigaku* 20, 205-7 (1960).

²⁰⁴ Sefaro Yomogita and Takashi Arima, *Kagaku Keisatsu Kenyusho Hokoku* 13, 36-7 (1960).

URACIL MUSTARD; 5-{*bis* (2-CHLOROETHYL)AMINO}URACIL, DOPAN; 5-*bis* (2-CHLOROETHYL)AMINO- β -METHYLURACIL), AND CHLORAM-BUCIL, 4{*p*-[*bis*(2-CHLOROETHYL)AMINO]PHENYL}BUTYRIC ACID

The above uracils are determined by reaction with 4-(*p*-nitrobenzyl)-pyridine.²⁰⁵ Proteins in plasma samples are precipitated by acetone and the ethanol is increased somewhat. Another reagent for uracil mustard is 8-hydroxyquinoline.²⁰⁶

Procedure—By 4-(*p*-nitrobenzyl)pyridine. Take 1 ml. of the sample containing 0.01-0.07 mg. of the drug in 5% dimethylacetamide in ethanol or 0.9% sodium chloride solution. Add 1 ml. of phthalate buffer for pH 4. Next add 1 ml. of 5% solution of 4-(*p*-nitrobenzyl)pyridine solution in acetone and 1 ml. of 0.9% sodium chloride solution. Dilute to 4 ml. with 95% ethanol and heat at 80° for 20 minutes. Chill in ice. Add 0.1 ml. of 5.6% solution of potassium hydroxide in 90% ethanol and dilute to 5 ml. with 95% ethanol. Read in 2-3 minutes at 600 $m\mu$ against a reagent blank, or in the case of plasma samples, at once.

Uracil mustard. By 8-hydroxyquinoline. Take up a 200-mg. sample in 95% ethanol and dilute to 100 ml. Mix 20 ml. of this solution with 20 ml. of water. Add 1 ml. of 5% solution of acetic acid and mix. Add 6 ml. of a 2% solution of 8-hydroxyquinoline and mix. Add 3 ml. of 10% sodium carbonate solution and dilute to 50 ml. After 2.5 hours, read at 466 $m\mu$ against a reagent blank.

1-PIPEROYLPIPERIDINE, PIPERINE

Piperine is the major sharp principle of pepper. By sulfuric acid hydrolysis, the methylenedioxy group is converted to formaldehyde and determined by chromotropic acid.²⁰⁷ Spectrophotometrically, it is read at 345 $m\mu$.²⁰⁸ However piperettine is also present, has a maximum at 364 $m\mu$ and may modify the reading. Correction can be made.

²⁰⁵ Oleta Klatt, A. C. Griffin and John S. Stehlin, Jr., *Proc. Soc. Exptl. Biol. Med.* **104**, 629-31 (1960); H. G. Petering and G. J. Van Giessen, *J. Pharm. Sci.* **52**, 1159-62 (1963).

²⁰⁶ American Pharmaceutical Association Foundation, *J. Pharm. Sci.* **53**, 1232-4 (1964).

²⁰⁷ *Official Analytical Methods*, American Spice Trade Association, Method 13, p. 32 (1960).

²⁰⁸ Harold J. Fagen, Eugene P. Kolen and Ralph V. Hussong, *J. Agr. Food Chem.* **3**, 860-2 (1955).

Procedure—*Pepper*. Extract a 0.5-gram sample, ground to pass a 20-mesh sieve, in 100 ml. of 95% ethanol. Boil for 3 hours, swirling occasionally. Cool, and dilute to 250 ml. with 95% ethanol. Filter or centrifuge suitable aliquots.

Colorimetric. Add 1-ml. aliquots to flasks A and B, and 1 ml. of 95% ethanol to flask C. Add 0.5 ml. of fresh aqueous 25% chromotropic acid solution to flasks A and C. Add 10 ml. of concentrated sulfuric acid to each and swirl. Heat at 100°, stoppering loosely for 1-2 minutes, then tightly. Cool to room temperature and dilute to 25 ml. with concentrated sulfuric acid. Mix thoroughly and read at 580 m μ . Flask C is the reagent blank for flask A, concentrated sulfuric acid for flask B.

$$\% \text{ piperine} = 10.81(A_1 - A_2)/\text{weight of sample}$$

Photometric. Dilute a 3-ml. aliquot of the ethanol extract to 100 ml. with 95% ethanol. Read at 343 m μ against 95% ethanol.

To determine piperettine, read at 364 m μ . Then correct the reading for each to apply it to the standard curve.

$$\text{Per cent of piperine} = 1.56(14.40A_{343} - 11.16A_{364})$$

$$\text{Per cent of piperettine} = 1.56(12.45A_{364} - 7.641A_{343})$$

In the ultraviolet. *Oleoresin of pepper*. Dissolve a 0.25-gram sample in chloroform and dilute to 100 ml. Dilute a 0.5-ml. aliquot to 100 ml. with chloroform. Read at 345 m μ against chloroform.

PIPERETTINE

For piperettine in the ultraviolet, see piperine.

CYCLOSERINE, D-4-AMINO-3-ISOXAZOLIDONE

See Pyrazinamide, page 496.

VITAMIN B₆

Vitamin B₆ may be *pyridoxine* (Vol. IV, p. 272-277), *pyridoxal*, or *pyridoxamine*.²⁰⁹ They are determined as phenols by reaction with 2,6-dibromoquinonechlorimide.

²⁰⁹ A. W. Indemans and H. E. J. Rademakers, *Pharm. Weekblad* 95, 377-93 (1960)

Pyridoxine, pyridoxal, and pyridoxamine are separated by a sulfonic acid ion-exchange resin.²¹⁰ Thereafter, pyridoxine is oxidized to pyridoxic acid by potassium permanganate. The pyridoxic acid is then converted to the pyridoxic acid lactone, since the greater fluorescence of the lactone permits its measurement in the presence of pyridoxine, pyridoxal, and pyridoxamine.²¹¹

Pyridoxamine undergoes a transamination reaction with glyoxylate, which produces a pyridoxal product, which, in turn, is reacted with potassium cyanide to produce pyridoxal cyanide.²¹² The reaction between cyanide and pyridoxal requires 2 hours for completion. Readings are made at pH 9.5 with 358 m μ activating wave length and 435 m μ fluorescent wave length. Fluorescence of pyridoxal and pyridoxamine are negligible at these wave lengths. Beer's law is followed for 0.000001-0.001 mg. of pyridoxamine per ml. 4-Pyridoxic acid lactone exhibits a fluorescent spectrum similar to that of pyridoxal cyanide.

Pyridoxine is determined by use of the indophenol reaction with *N,N*-dimethyl-*p*-phenylenediamine hydrochloride and sodium hypochlorite.²¹³ The pyridoxine in multivitamin preparations must first be separated by paper chromatography, using the upper layer of 4:1:5 butanol-aqueous ammonia-water.

With 2,6-dichloroquinonechlorimide in butanol, 0.5 mg. of pyridoxine can be determined in the presence of 5,000 I.U. of vitamin A, 500 I.U. of vitamin D₂, 1 mg. of vitamin E, 2 mg. of vitamin B₁, 2 mg. of vitamin B₆ phosphate, 20 mg. of niacin, 1 mg. of pantothenic acid calcium salt, 0.001 mg. of vitamin B₁₂, and 75 mg. of vitamin C.²¹⁴ The accuracy of the determination of vitamin B₆ by development of a blue color with 2,6-dichloroquinone chlorimide is improved by the use of a 1% solution of sodium sulfate to prevent interference from lead.²¹⁵

²¹⁰ P. A. Hedin, *J. Agr. Food Chem.* **11**, 343-5 (1963).

²¹¹ Akiji Fujita, Kanji Matsuura and Kohei Fujino, *Vitamins* (Japan) **6**, 389-33 (1953); Kohei Fujino, *ibid.* **6**, 876-81 (1953); Daisuke Fujita, *ibid.* **7**, 502-7 (1954); Kohei Fujino, *ibid.* **7**, 638-43 (1954); Kohei Fujino, *ibid.* **7**, 644-7 (1954); Kohei Fujino, *ibid.* **8**, 239-41 (1955); Kohei Fujino, *ibid.* **9**, 100-9 (1955); Douglas J. Hennessy, Anne M. Steinberg, George S. Wilson and William P. Keaveney, *J. Assoc. Offic. Agr. Chemists* **43**, 765-8 (1960).

²¹² Edward W. Toepfer, Marilyn MacArthur Polansky and Elizabeth M. Hewston, *Anal. Biochem.* **2**, 463-9 (1961).

²¹³ Konosuke Murai, *Arch. Prac. Pharm. Japan* **21**, 124-7 (1961).

²¹⁴ Tsutou Aihara and Kazuo Sato, *Vitamins* **8**, 164-5 (1955); L. Campanioli and M. Giannini, *Farmaco* (Pavia) *Ed. prat.* **11**, 229-34 (1956); E. V. Bogdanova, *Vsesoyuz. Nauch-Issledovatel. Vitamin Inst.* **6**, 249-51 (1959).

²¹⁵ E. Benhamou and R. Lacroix, *Ann. biol. clin.* **16**, 78-83 (1958); E. V. Bog-

The direct fluorescence of pyridoxine is at 405 m μ .²¹⁶ For reading it in the ultraviolet in the presence of thiamine and nicotinamide, see page 437. Acetone extracts of whole blood can be read fluorescently for pyridoxine, pyridoxal, and pyridoxamine.²¹⁷ Metabolism of vitamin B₆ in urine gives 4-pyridoxic acid.²¹⁸

Sample—Enriched flour. To prepare the activated Decalso, wash 200 grams of 60-80 mesh grade by decanting three times with 1-liter portions of water. Pour off the extreme fine resin with the wash water. Pour 3 liters of boiling 1:59 hydrochloric acid on the resin, and while stirring mechanically, keep the mixture at the boil for 10 minutes. Discard the supernatant liquid and repeat the hot acid treatment 5 additional times. This removes iron and considerable aluminum from the resin.

The ion-exchange tubes are 0.7 \times 15 cm. with a 50-ml. reservoir at the top and a 0.04 \times 3-mm. capillary at the bottom. Place a plug at folded gauze in the bottom. Add water until the reservoir is about half full. Add the activated Decalso to within about 1 cm. from the top of the tube. Cover with another gauze plug, drain the reservoir and retain the water in the tube.

Mix a 5-gram sample with 25 ml. of water to form a smooth paste. Add 50 ml. of water and 1 ml. of 1:9 sulfuric acid. Heat at 100° for 15 minutes with frequent mixing. Continue the heating 15 minutes longer and cool to 50°. Add 2 ml. of a solution containing 544 grams of sodium acetate trihydrate per liter. Add 5 ml. of freshly prepared 6% solution of Mylase P. Incubate at 50° overnight. Cool and dilute to 100 ml. Filter, discarding the first 15 ml. of filtrate.

Add 10, 15, or 20 ml. of the extract to the reservoir of the ion-exchange tube and let it flow through by gravity. Wash with 10 ml. and 10 ml. of water. Add 10 ml. of sulfuric acid containing 6 ml. of concentrated acid per liter. When the first 10 ml. of acid reaches the upper surface of the resin, add an additional 10 ml. When this drains, add 5 ml. of acid. Dilute the effluent to 25 ml. with 1:160 sulfuric acid and mix well. Develop as pyridoxic acid lactone.

danova, *Trudy Vsesoyuz. Nauch.-Issledovatel. Vitamin Inst.* **6**, 249-51 (1959); V. A. Dovyatnin and G. A. Federova, *Tr. Uses. Nauchn-Issled. Vitamin Inst.* **7**, 95-102 (1961).

²¹⁶ Fujio Obo and Yoshihisha Tomohiro, *Agaka to Seibutsugaku* **34**, 127-9 (1955); Mia Bercovici, *Rev. chim.* (Bucharest) **9**, 335-7 (1958).

²¹⁷ David B. Coursin and Virginia C. Brown, *Proc. Exptl. Biol. Med.* **98**, 315-18 (1958); Dewayne Roberts and Morris Friedkin, *J. Biol. Chem.* **233**, 483-7 (1958)

²¹⁸ V. A. Bogdanova, *Vopr. Pitaniya* **18**, No. 5 46-50 (1959).

Fresh bread prepared from enriched flour. Follow the procedure under "Enriched flour," starting at the beginning. Substitute the following paragraph for the paragraph starting with "Mix a 5 gram sample . . ." and ending with "Add 2 ml. of 54.4% sodium acetate solution":

Take a 50-gram sample containing a representative portion of crust, and crumb. Add to 300 ml. of water in a blender and work to a smooth paste. Transfer 50 grams of the paste to a 100-ml. flask, using 25 ml. of water. Add 1 ml. of 1:9 sulfuric acid and heat at 100° for 30 minutes. Cool to 50° and add 2 ml. of the sodium acetate solution. Complete as for enriched flour.

Multivitamin preparations. Separate pyridoxine by paper chromatography using the upper layer of a 4:1:5 mixture of butanol-ammonium hydroxide-water as the developing solvent. After extraction, develop with *N,N*-dimethyl-*p*-phenylenediamine hydrochloride and sodium hypochlorite.

Procedure—*As pyridoxic acid lactone.* To prepare the complexing reagent, dissolve 200 grams of the sodium salt of ethylenediamine tetraacetic acid and 200 grams of potassium sodium tartrate in water, and dilute to 1 liter. Stir the solution for 20 minutes with each of four 20-gram portions of decolorizing carbon and filter with suction after each treatment. This prevents the precipitation of the hydrated oxides of manganese and aluminum at pH 9.5. After separation of interferences by chromatography, pipet two 10-ml. aliquots of the effluent into tubes graduated at 22 ml. Add 1 ml. of isopropyl alcohol to each and cool in an ice-water bath to 0-2°. Add a 2-ml. portion of ice cold 2% potassium permanganate solution to each and mix. This oxidizes the pyridoxine to pyridoxic acid. To remove excess permanganate, after 50 seconds, add with mixing 0.5 ml. of 3% hydrogen peroxide prepared before use by diluting the 30% grade.

Reserve one tube for lactonization. Heat the remaining tube, which serves as a blank, at 100° for 2 minutes to drive off oxygen so that the effervescence does not interfere with the reading. Cool the blank in ice water to 0-2°, and add 2 ml. of 3:1 hydrochloric acid and 2 ml. of complexing reagent with mixing. Dilute to 22 ml. with 1:1 aqua ammonia. Determine the blank fluorescence with 15 ml. of this solution.

To the tube reserved for lactonization, add 2 ml. of 3:1 hydrochloric acid and heat at 100° for 12 minutes. Cool in ice water to 0-2° and add

2 ml. of complexing mixture. Dilute to 22 ml. with 1:1 aqua ammonia and read a 15-ml. portion fluorometrically as for determination of thiamine. Calculate as follows:

$k[(d - d_o)/ds] = \text{mmg. of pyridoxine hydrochloride in a 10 ml. aliquot}$
in which k is the proportionality constant of 2.8; d is the galvanometer deflection of the sample; d_o is the galvanometer deflection of the blank; and d_s is the galvanometer deflection of a reference working standard prepared from 0.1 mmg. of quinine per ml. in 1:350 sulfuric acid.

By transamination to pyridoxal and reaction with cyanide. Carry out the procedure in the dark, as pyridoxal and pyridoxamine are light sensitive. To a 5-ml. solution containing about 0.001 mg. of pyridoxamine per ml., add 10 ml. of 17.4% dibasic potassium phosphate adjusted to pH 7.4 with 1:10 hydrochloric acid. Add 1 ml. of 4.7% potassium alum solution and 1 ml. of 6% sodium glyoxylate solution. Heat for 15 minutes at 100°. Cool to room temperature and add 1 ml. of 3.2% potassium cyanide solution. Adjust the pH to 7.2-7.4 and dilute to 50 ml. Warm for 2.5 hours at 50°. Adjust the pH to 9.5 and read the fluorescence at 435 m μ with an activating wave length of 358 m μ .

By N,N-dimethyl-p-phenylenediamine hydrochloride and sodium hypochlorite. To a 2-ml. sample containing 0.01-0.1 mg. of pyridoxine, add 2 ml. of Sorensen buffer at pH 8, add 0.5 ml. of 0.05% N,N-dimethyl-p-phenylenediamine hydrochloride solution, 2 ml. of isobutanol, and 0.5 ml. of 0.2% sodium hypochlorite solution. Shake vigorously for 5 minutes and let stand at room temperature for 5 minutes. Dilute the organic layer with 0.2 ml. of anhydrous ethanol and read at 625 m μ .

By 2,6-dichloroquinone chlorimide. Dilute the sample with 10% acetic acid or extract with 10% acetic acid and adjust the final concentration of pyridoxine to about 0.16 mg. per ml. at pH 3. To a 10-ml. aliquot of the sample, add 10 ml. of 5% silver nitrate solution and 10 ml. of 0.4% sodium hydroxide solution. Adjust the pH to 4.5-5.5. Centrifuge off the precipitate and dilute the supernatant liquid to 100 ml. with water. Adjust the pH of a 5-ml. aliquot to pH 3 with 1:1 hydrochloric acid. Add 0.2 gram of acidic clay and centrifuge to sediment the clay. Treat the clay with 10 ml. of monobasic potassium phosphate-sodium hydroxide reagent and 5 ml. of 0.025% 2,6-dichloroquinone chlorimide solution in butanol. Measure the blue color formed in the butanol layer.

Alternatively,²¹⁹ prepare a buffer solution containing 46.85 ml. of 0.8% sodium hydroxide solution, 50 ml. of 2.7% potassium dihydrogen phosphate solution, and water to dilute to 200 ml. As reagent, dissolve 20 mg. of dibromoquinone chlorimide in 320 ml. of isobutanol.

Mix 1 ml. of sample containing about 0.2 mg. of vitamin B₆ with 4 ml. of buffer. Separate 2 ml. of this and add 5 ml. of reagent. Heat at 50° for 1 minute and let stand for 10 minutes. Shake for 1 minute and heat for 10 minutes at 50°. Separate, and filter the isobutanol layer. Add 20-50 mg. of anhydrous sodium sulfate to dehydrate. Read after 2-3 hours.

BARBITURIC ACID AND BARBITURATES, BARBITALS

Ultraviolet methods are based on the fact that 5,5'-disubstituted barbiturates exist in three forms in solution. The un-ionized form in acid solution has no selective light absorption in the range of 230-270 m μ . The first ionized form at pH 9.8-10.5 has an absorption maximum at 240 m μ but no minimum in the range of 230-270 m μ . A second ionized form at pH 13-14 has an absorption maximum at 252-255 m μ with a minimum at 234-237 m μ .²²⁰ The absorption maximum at pH 10 at 240 m μ is not specific for barbiturates. The barbiturates can be detected at pH 13 and 10, and the spectra are characteristic.

Barbiturates are read at 260 m μ in 4% sodium hydroxide solution before and after addition of ethylenediamine hydrochloride.²²¹ Blood is extracted with butyl ether. This extract is extracted with two portions of borax and then with sodium hydroxide solution. Most of the barbiturates of lowest potency are in the first borax extract. The barbiturates of highest potency, including amobarbital, phenobarbital, and secobarbital, are present in the sodium hydroxide. The absorbance of the borax extract is read at 245, 240, and 235 m μ ; the absorbance of the sodium hydroxide extract is read at 260, 255, 250, and 240 m μ . Ethylenediamine hydrochloride is added and the solution is read at 260, 245, 235, and 230 m μ . A derived equation is applied to determine the nature and amount of barbiturate present in the blood.

²¹⁹ Atsushi Watanabe and Mieko Horikawa *Ann. Rept. Takeda Research Lab.* **8**, 110-13 (1949); cf. Atsushi Watanabe and Toyoko Murakimi, *ibid.* 103-10.

²²⁰ P. M. G. Broughton, *Biochem. J.* **63**, 207-13 (1956); I. Mathäuser, *Wien. Klin. Wochschr.* **69**, 913-5 (1957).

²²¹ George W. Stevenson, *Anal. Chem.* **33**, 1374-8 (1961).

Chloroform is commonly used to extract barbiturates from blood.²²² The interference of salicylate is removed by washing the chloroform extracts of blood with phosphate buffer. Sulphanilamides and their metabolic products interfere. Megimide, β -ethyl- β -methylglutarimide, does not interfere.

In a modified procedure for blood samples, barbiturates are forced into chloroform by removal of water and protein with anhydrous sodium sulfate. Hydrochloric acid is added to increase the solubility of the barbiturates in chloroform. The volume of chloroform needed is small by this technic and a better re-extraction into water is therefore obtained.²²³ Sulphonamides and related compounds cause high results. Salicylates give low results and may mask the barbiturates.

Barbiturates may be read in boric acid-potassium chloride solution at pH 9.5.²²⁴ Stearates do not interfere. When barbiturates are read directly below 240 m μ in alkaline solution, other drugs such as bemigrade, β -ethyl- β -methylglutarimide, cause a high absorption.²²⁵ To remove this, incubate at 38° for 2 hours. Such hydrolysis also destroys carbromal. If salicylates are present, a correction must be applied.²²⁶ Phenobarbital and methocarbamol are determined simultaneously in borate buffer at pH 9.5.²²⁷

Barbiturates and thiobarbiturates converted to violurates by nitrite absorb at 520 m μ and 570 m μ respectively. The optimum pH for reading barbiturates is 5.0-5.2; for thiobarbiturates, 4.7-4.9.²²⁸ Thiobarbiturates extracted with chloroform, can be read at 283 m μ in the presence of barbiturates and salicylates.²²⁹ Barbiturates give a suitable color for

²²² P. M. G. Broughton, *Biochem. J.* **63**, 207-13 (1956); R. Richterich, *Clin. Chim. Acta* **3**, 183-96 (1958); D. Westerink and J. H. Glerum, *Pharm. Weekblad* **99**, 657-64 (1964).

²²³ R. Askevoid and F. Loken, *Scand. J. Clin. & Lab. Invest.* **8**, 1-5 (1956).

²²⁴ Leland N. Mattson, *J. Am. Pharm. Assoc.* **43**, 22-4 (1954).

²²⁵ P. M. G. Broughton, *Lancet*, **i**, 1963, 1266-7.

²²⁶ Regina Guzak and Wendell T. Caraway, *Am. J. Med. Technol.* **29** (4), 231-9 (1963).

²²⁷ F. Pico and J. Bastus, *Galencia Acta* (Madrid) **15**, (5), 383-91 (1962).

²²⁸ N. S. Drozdov and V. P. Krylov, *Khim. Nauka i Prom.* **4**, 798-9 (1959); *Izvest. Vses. S'ezh. Ucheb. Zavedenii Khim. i Khim. Tekhnol.* **3**, 476-9 (1960); *Zh. Analit. Khim.* **15**, 248-9 (1960); *Doklady Akad. Nauk. USSR* **135**, 1135-8 (1960); cf. V. I. Lobanov, *Aptekhn. Delo* **11**, No. 3, 66-7 (1962); *Sudebno-Med. Ekspertiza Min. Zdrovookhr. SSSR* **6**, 39-44 (1963).

²²⁹ Louis A. Williams, Allen T. Hardy, Jerry S. Cohen and Bennie Zak, *J. Med. Pharm. Chem.* **11**, 609-15 (1960).

reading in the presence of cobalt acetate.²³⁰ The method is applicable to barbitone, phenobarbitone, amylobarbitone, butobarbitone, and quinalbarbitone. Coatings and lubricants of the tablets do not interfere.

Phenobarbital is separated from diphenylhydantoin in blood samples by partition in a cyclohexane-butanol mixture. Both drugs are read in the aqueous phase in the ultraviolet.²³¹ The butanol increases the extractability of diphenylhydantoin into cyclohexane. However, since phenobarbital is also extracted, the amount of butanol used is critical. Acetylsalicylic acid, trimethadione, and paramethadione do not interfere. Nirvanol or 5-ethyl-5-phenyl hydantoin, the demethylated metabolite of Mesantoin, 3-methyl-5-ethyl-5-phenyl hydantoin, interferes with diphenylhydantoin. Error from substances normally encountered in blood is 1.5 microgram per ml. for diphenylhydantoin and 0.5 microgram per ml. for phenobarbital. Beer's law is followed. Up to 0.1 mg. of the diphenylhydantoin and phenobarbital are extracted from 1-2 ml. of acidified serum and partitioned between chloroform and a borate buffer for pH 8.8 for reading.²³²

Phenobarbital and mannitol hexanitrate are read in the ultraviolet in ammonium hydroxide solution after extraction of the sample with glacial acetic acid.²³³ The range of this method is up to 5 mg. per 100 ml. Various methods have been compared.²³⁴ The differential ultraviolet method is accurate, precise, and substantially free from interference. Dealkylation of phenobarbital and pentobarbital in sulfuric acid causes a shift in ultraviolet absorbance.²³⁵

In the determination of barbiturates in liver by differential absorption at 240 m μ in 0.5% ammonium hydroxide or in acid, interference has been encountered from an unknown phenolic-like compound in the liver of deceased persons and in the urine of drunken persons.²³⁶ This compound may add 0.001-0.004 mg. to the barbiturate readings per 100 grams of liver tissue and must be separated by paper chromatography in a *n*-butanol/5 *N* ammonia system. In such a system, the unknown compound runs at R_F 0.95. Sodium secobarbital is determined as barbituric acid by bromination with potassium bromate and potassium bromide in a

²³⁰ Vishnu Das Gupta, *Indian J. Pharm.* **25**, 161-3 (1963).

²³¹ Gabriel L. Plaa and Charles H. Hine, *J. Lab. Clin. Med.* **47**, 649-57 (1956).

²³² Ole Svensmark and Paul Kristensen, *ibid.* **61**, 501-7 (1963).

²³³ Evelyn Sarnoff, *J. Assoc. Offic. Agr. Chemists* **41**, 493-5 (1958).

²³⁴ F. Fishler, L. F. Worrell and J. E. Sinsheimer, *J. Pharm. Sci.* **51**, 695-8 (1962).

²³⁵ Theodore L. Brown, *ibid.* **52**, 274-5 (1963).

²³⁶ A. S. Curry, *Nature* **176**, 877-8 (1958).

solution buffered at pH 6.8.²³⁷ Error is reduced by large dilutions of the sample. Beer's law is followed for 0.5-2 mg. of sodium secobarbital. Total barbiturates may also be determined as barbituric acid by bromination.

Phenobarbital at 0.05-0.15 mg. per ml. is determined nephelometrically by adding 1.9% mercuric perchlorate solution.²³⁸

Barbituric acid derivatives monosubstituted in position 5 and 3,5-dioxypyrazolidine containing an activated hydrogen atom are determined with xanthidrol.²³⁹ This can be applied to Butazoline in the presence of aminopyrine. For analysis of reopirin dragees for Butazolidine, the sugar coating must be removed first. The sensitivity is to 0.15 mg. per ml.

Procedure—*In the ultraviolet. Whole blood.* Extract a 5-10 ml. sample with three 10-ml. portions of chloroform. Wash extracts containing salicylic acid or sulphonamides with two 5-ml. portions of 0.5 *M* potassium-sodium phosphate buffer at pH 7.4. Filter the combined chloroform extracts and wash the filter paper with a little chloroform. Extract the chloroform with 5 ml. of 1.8% sodium hydroxide solution by vigorous shaking for 1 minute. Allow the layers to separate and clarify the aqueous layer by centrifuging.

Heat the clear 1.8% sodium hydroxide extract containing up to 0.1 mg. of barbiturate at 100° for 15 minutes. Cool rapidly in cold water and dilute to 5 ml. with water. Add 2 ml. of the extract to 2 ml. of 1.8% sodium hydroxide solution and add another 2 ml. portion to 2 ml. of 0.6 *M* boric acid-potassium chloride. Read both solutions at 227 and 265 $m\mu$ against sodium hydroxide solution and the borate blank solution, respectively.

The criteria for the spectrophotometric determination of barbiturates are as follows: maximum at 238-240 $m\mu$ in borate; maximum at 252-255 $m\mu$ and a minimum at 234-237 $m\mu$ in sodium hydroxide; isosbestic points at 227-230 $m\mu$ and at 247-250 $m\mu$.

Whole blood. Amobarbital, pentobarbital and secobarbital. To prepare the borate buffer, dissolve 19.05 grams of sodium borate decahydrate in water and dilute to 1 liter. The pH should be approximately 9.2 at 25°.

Rapidly mix 5 ml. of blood and 25 ml. of *n*-butyl ether by inverting approximately 200 times. Centrifuge until the ether layer is free of

²³⁷ George E. Keepel, *J. Assoc. Offic. Agr. Chemists* **38**, 630-5 (1955).

²³⁸ Kazimierz Kalinowski and Henryk Baran, *Acta Polon. Pharm.* **15**, 327-31 (1958).

²³⁹ D. Dalev and I. Gagawzov, *Nauchni. Tr. Visshiya Med. Inst., Sofiya* **7**, No. 5, 119-24 (1960).

aqueous drops. Mix 20 ml. of the ether extract with 5 ml. of borate buffer by inverting rapidly 100 times. Centrifuge. Remove all of the buffer with a pipet, expelling air while passing the pipet through the ether layer. Label this portion Borax 1. Add another 5-ml. portion of borate buffer to the ether layer and repeat the above procedure, starting at "by inverting rapidly 100 times." Label this portion Borax 2.

Add 5 ml. of 4% sodium hydroxide solution to each borate buffer extract and equilibrate by inverting rapidly 100 times. Centrifuge. Pipet 1 ml. of the sodium hydroxide solution from the sample tubes using 1000 microliter micropipets and a mouth tube. Occlude the top of the pipe while inserting it through the ether layer, expel air to remove ether from the tip, and wipe ether from the outside of the pipet with tissue after filling.

Read the sodium hydroxide solution at 260, 255, and 240 $m\mu$. Add 250 microliters of 34.8% ethylenediamine hydrochloride solution to the sodium hydroxide solution. Mix by filling and expelling the contents of a 1000-microliter pipet. Read at 260, 245, 240, 235, and 230 $m\mu$. Calculate barbiturate as follows:

$$\text{barbituric acid, mg. } \% = k_p \times A_{260(a-b)}$$

where $A_{260(a-b)}$ is $A_{260a} - 1.25 \times A_{260b}$, and k_p is 7.43.

Read the absorbances of the Borax 1 and Borax 2 solutions at 245 and 240 and 235 $m\mu$ against borax washes of fresh ether. Calculate the ratios as follows:

$$\text{ratio 1} = \frac{A_{240d}}{A_{260(a-b)}}$$

$$\text{ratio 2} = \frac{A_{240c}}{A_{240d}}$$

The values for these ratios for pure barbiturates are as follows:

For determination of more polar barbiturates including phenobarbital,

$$\text{barbituric acid, mg. } \% = k_d (A_{240c} - A_{240d})$$

in which k_d is a constant for the particular barbiturates as found in Table 30.

For barbiturates of intermediate polarity,

$$\text{barbituric acid, mg. } \% = k_s (A_{240c} + A_{240d})$$

Whole blood. Total barbiturates. Follow the procedure for determination of aminobarbital, pentobarbital, and secobarbital, extracting the butyl ether with 4% sodium hydroxide solution only. Use a 5-fold volume

TABLE 30. CONSTANTS FOR IDENTIFICATION AND DETERMINATION OF BARBITURATES

<i>Barbituric Acid Order of Polarity</i>	<i>Constants for Identification</i>		<i>Constants for Determination</i>			
	<i>Ratio 1</i>	<i>Ratio 2</i>	<i>k_p</i>	<i>k_i</i>	<i>k_d</i>	<i>k_s</i>
Hexethal	0.10	1	6.30	5.67	...	32
Sigmodal	0.12	1	10.8	9.33	...	34
Secobarbital	0.15	1.05	7.35	6.31	...	24
Pentobarbital	0.25	1.11	7.49	5.84	...	14.3
Amobarbital	0.33	1.18	7.45	5.31	...	10.3
Butallylonal	0.41	1.28	13.4	9.25	...	15.3
Allyl, <i>sec</i> -butyl	0.51	1.29	10.7	6.49	72	9.15
Butabarbital	0.87	1.50	11.8	5.26	27	5.41
Butethal	0.86	1.51	12.0	5.30	27.5	5.56
Allyl, isobutyl	0.93	1.52	13.8	6.01	28.6	5.89
Cycloheptenyl, ethyl	1.19	1.6	17.2	6.36	23.2	5.52
Nostal	1.44	1.7	22.1	8.85	32.6	7.61
Aprobarbital	2.0	2.0	25.4	6.09	12.5	4.28
Cyclobarbital	3.2	2.5	41	6.60	9.68	4.15
Vinbarbital	2.8	2.6	...	5.80	8.30	3.66
Probarbital	2.8	2.7	...	5.34	7.86	3.66
Allyl, phenyl	3.7	3.0	...	7.40	8.25	4.13
Dial	5.6	3.5	...	5.87	6.64	3.60
Phenobarbital	6.5	4.4	...	6.51	5.69	3.60
Methyl, phenyl	14	7.9	...	6.83	4.65	3.60
Barbital	17	7.9	...	5.90	4.35	3.38

of butyl ether for extraction of blood. Calculate barbiturates as follows:

$$\text{barbituric acid, mg. } \% = 4k_i \frac{V_{\text{NaOH}}}{V_{b.e.}} (A_{260e} - 1.25A_{260i})$$

in which k_i is a constant given in Table 30. If the identity of the barbiturate is not known, use the constant for pentobarbital.

V_{NaOH} and $V_{b.e.}$ = volumes of sodium hydroxide and butyl ether used in the sodium hydroxide extraction of the butyl ether.

Serum. Add a 3-ml. sample to 15 ml. of chloroform. Add 2 grams of sodium sulfate and 0.1 ml. of concentrated hydrochloric acid. Shake and add 2 grams more of anhydrous sodium sulfate. Shake. The sodium sulfate and proteins should appear as hard lumps in the water phase and the chloroform should be free from water droplets. If the chloroform contains water, shake with an additional 2 grams of anhydrous sodium sulfate.

Filter. Vigorously shake 5 ml. of the filtrate with 5 ml. of borate buffer at pH 10 for 3 minutes. Centrifuge and read the aqueous layer at 239 $m\mu$ against a reagent blank. Add 0.05 ml. of concentrated hydrochloric acid to both the sample and blank and read. The difference in reading $\times 10.6 =$ mg. of sodium barbiturate.

Blood. Phenobarbital and diphenylhydantoin simultaneously. To prepare the carbonate buffer at pH 11, dissolve 8.4 grams of sodium bicarbonate and 3.6 grams of sodium hydroxide in 1 liter of water. To a mixture of 0.5 gram of sodium bicarbonate and 0.2 ml. of 10% sodium hydroxide solution, add 5 ml. of oxalated whole blood. The pH should be 8.5. Add 20 ml. of cyclohexane and 1 ml. of *n*-butanol. Stopper, shake for 5 minutes and centrifuge at 2500 rpm. for 5 minutes. The diphenylhydantoin is in the organic phase and approximately 90% of the phenobarbital remains in the aqueous phase.

Using the aqueous phase for determination of phenobarbital, aspirate any cyclohexane remaining. Adjust the pH to 5.5-6.0 by adding, 1 ml. at a time, approximately 2 ml. of 25% acetic acid. When no more carbon dioxide is being evolved and no more sodium bicarbonate is visible at the bottom of the tube, add 35 ml. of chloroform. Shake for 5 minutes. Centrifuge and decant the aqueous phase above the resulting blood clot. Break through the blood clot. Filter the chloroform phase. Add 30 ml. of 1:3 sulfuric acid to the chloroform phase, shake for 5 minutes and centrifuge. Aspirate the acid phase. Shake 25 ml. of the chloroform phase with 5 ml. of 1.2% sodium hydroxide solution for 5 minutes. Centrifuge at 3000-4000 rpm. to eliminate turbidity. If an emulsion forms, break it with a glass rod and recentrifuge. To prepare the reagent blank, run 5 ml. of water through the above extraction procedure. The carbonate buffer is the hydantoin blank and the 1.2% sodium hydroxide solution is the barbiturate blank.

Read a 3-ml. aliquot against the reagent blank at 260, 250, and 240 $m\mu$. Add 0.5 ml. of saturated potassium bicarbonate solution to the sample and blank to raise the pH to 10-10.5. Read at 260, 250, and 240 $m\mu$. Multiply the second readings by 1.17 to correct for dilution and subtract from the first readings. Barbiturate is present if a maximum positive optical difference occurs at 260 $m\mu$, a minimal difference at 250 $m\mu$, and a maximum negative difference at 240 $m\mu$. Calculate phenobarbital from the difference at 260 $m\mu$, 10 mcg./ml. = 0.205 optical density units.

Add the cyclohexane phase of the blood extract containing the

diphenylhydantoin to 10 ml. of 1:10 hydrochloric acid, stopper, and shake for 5 minutes. Centrifuge. Add 15 ml. of the cyclohexane phase to 5 ml. of a carbonate buffer at pH 11, discarding the acid wash. Shake for 5 minutes and centrifuge. Aspirate, discard the cyclohexane layer, and determine hydantoin in the aqueous phase. Prepare a reagent blank as described under barbiturates.

Read a 3-ml. aliquot against the reagent blank at 260, 250, 240, and 235 $m\mu$. Add 0.5 ml. of 10% sodium hydroxide solution to adjust the pH to 12.5 and read again at the same wave lengths. Multiply the second readings by 1.17 to correct for dilution, and subtract from the first readings. If a detectable amount of phenobarbital is present, the spectrum will shift in proportion to the amount present and will exhibit the change in readings at 260, 250, and 240 $m\mu$ as described in the phenobarbital section. Calculate the absorbance due to phenobarbital in the carbonate buffer at pH 11 at 260 and 235 $m\mu$ as follows:

O.D. phenobarbital at 260 $m\mu$ = $0.668 \times$ O.D. difference at 260 $m\mu$

O.D. phenobarbital at 235 $m\mu$ = $2.12 \times$ O.D. difference at 260 $m\mu$

Subtract the calculated optical densities from those obtained in the first readings. For hydantoin, 10 mcg./ml. = 0.196 optical density units.

Blood and urine. Dilute a 0.2-ml. sample containing 0.004-0.08 mg. of barbiturates to 0.5 ml. with pH 7.4 phosphate buffer. Extract with two 30-ml. portions of chloroform and shake the combined extracts with 6 ml. of 0.4% sodium hydroxide solution. Centrifuge, and add 4 ml. of the alkaline extract to 4 ml. of pH 10 borate buffer. Read at 240 $m\mu$. Adjust the pH to 2 with 5 drops of 1:5 hydrochloric acid. Read again at pH 2 and subtract the reading from that at pH 10.

Urine, gastric contents and liquid samples. Acidify a 10-25 ml. sample with sulfuric acid and extract with chloroform following the first procedure for whole blood listed in this section. Wash the combined chloroform extracts twice with 5 ml. of 0.5 *M* phosphate buffer at pH 7.4 before filtering. Follow the procedure for whole blood, listed first in this section, starting at "Heat the clear 1.8% sodium hydroxide extract . . .," except that, in this case, it is the phosphate buffer extracts.

Tissues. Homogenize a 1-5 gram sample with chloroform and follow the above procedure for urine, gastric contents, and liquid samples, starting at the beginning.

Tablets. Dissolve a sample equivalent to 30 mg. of phenobarbital in glacial acetic acid and dilute to 50 ml. with glacial acetic acid. Shake and filter, discarding the first few ml. of the filtrate. Dilute a 10-ml. portion of the filtrate to 50 ml. with water, shake and filter, discarding the first few ml. of the filtrate.

Prepare a blank by diluting 10 ml. of glacial acetic acid to 50 ml. with water and filter. Dilute 20 ml. of the standard, sample, and blank aliquots to 100 ml. with 1:9 ammonium hydroxide at room temperature. Read against the blank at 235, 240 and 270 $m\mu$. Determine phenobarbital as follows:

$$A_{\text{sample}} \times 12.5 \times \frac{\text{wt. phenobarbital}}{(A_{\text{std.}} \times \text{wt. sample})} = \% \text{ phenobarbital}$$

Calculate A (corr.) for sample and standard and substitute in the above formula:

$$A(\text{corr.}) = A_{240} + \frac{(A_{235} - A_{270})}{7 - A_{235}}$$

Phenobarbital tablets U.S.P. To prepare the boric acid-potassium chloride solution, dissolve 6.185 grams of boric acid and 7.455 grams of potassium chloride in water and dilute to 1 liter with water. To prepare a buffer at pH 9.5, add 34.33 ml. of 0.4% sodium hydroxide solution to 50 ml. of boric acid-potassium chloride solution and dilute to 100 ml. with water.

Dissolve a finely ground sample containing up to 25 mg. of phenobarbital in the pH 9.5 buffer and dilute to 250 ml. with the buffer. Mix, and let stand 10 minutes with occasional shaking. Filter, discarding the first 25 ml. of filtrate. Dilute a 10-ml. portion of the filtrate to 100 ml. with buffer. Mix, and read at 240 $m\mu$ against the buffer.

Phenobarbital elixir U.S.P. Dilute a 5-ml. sample to 100 ml. with pH 9.5 buffer as described under Phenobarbital Tablets U.S.P. Mix, and dilute a 5-ml. aliquot to 100 ml. with buffer. Read at 240 $m\mu$ against an elixir base without phenobarbital which has been treated in the same way as the sample.

Phenobarbital elixir "Not-U.S.P." Dilute a 5-ml. sample to 100 ml. with pH 9.5 buffer, as described under Phenobarbital Tablets U.S.P. Mix and dilute a 5-ml. aliquot to 100 ml. with ethanol. Evaporate a 5-ml. aliquot to dryness at 100° to remove interferences. Dissolve the residue in 100 ml. of buffer and read at 240 $m\mu$ against buffer. In the presence

of amaranth, treat a blank containing elixir base without phenobarbital in the same manner as the sample.

Ephedrine sulfate and phenobarbital capsules. Dissolve a sample containing up to 100 mg. of phenobarbital in 10 ml. of 2% sodium hydroxide solution, and extract with five 25-ml. portions of ether. Wash the combined ether extracts with three 15-ml. portions of water. Wash the water through 25 ml. of ether. Determine the ephedrine sulfate content of the ether extract (p. 115).

Discard the extracted aqueous alkaline sample. Dilute a second sample, containing up to 25 mg. of phenobarbital, to 250 ml. with the pH 9.5 buffer. Mix, and dilute a 10-ml. aliquot to 100 ml. with the buffer. Mix, and read at 240 $m\mu$. Correct the readings for ephedrine present as follows:

$$ODC = OD - (e \times 3.32)$$

in which ODC is the corrected optical density; OD is the observed optical density; e is the grams of ephedrine sulfate in the final 100 ml. dilution; and 3.32 is $E_{1\text{cm}}^{1\%}$ at 240 $m\mu$ for ephedrine sulfate at pH 9.5.

*Phenobarbital and pentobarbital.*²⁴⁰ Dissolve a sample containing 2.5-5 mg. of either phenobarbital or pentobarbital, or both, in 1 ml. of 0.5% sodium hydroxide solution. Mix with 9 ml. of concentrated sulfuric acid and heat at 100° for 30 minutes. Cool, and dilute to 50 ml. with water. Mix a 5-ml. aliquot with 5 ml. of concentrated ammonium hydroxide. Dilute to 100 ml. and read at 240 and 268 $m\mu$ against a reagent blank.

*Phenobarbitone and diphenylhydantoin (phenytoin).*²⁴¹ The mixture should be 1:3.8 to 1:10, preferably around 1:6.25. Dissolve a 290-mg. sample in 80 ml. of 0.2% sodium hydroxide solution and make up to 100 ml. with the sodium hydroxide solution. Dilute a 5-ml. aliquot to 200 ml. with water, let stand for 1 hour, and add 10 ml. of 4% sodium hydroxide solution.

Take a second 5-ml. aliquot, add 10 ml. of 4% sodium hydroxide solution, and heat at 100° for 1 hour. Cool, and add 150 ml. of water. Add 12.5 ml. of concentrated ammonium hydroxide to each aliquot and dilute to 250 ml. with water. Read each at 250 $m\mu$ against an appropriate

²⁴⁰ T. L. Brown, *J. Pharm. Sci.* **52**, 274-5 (1963).

²⁴¹ H. Sattler, *Pharm. Ztg. Ver. Apotheker-Ztg.* **109**, 584-7 (1964).

blank of 0.16% sodium hydroxide solution containing 5% of concentrated ammonium hydroxide solution.

The reading of the solution heated with alkali gives the phenytoin. The reading of the unheated solution less that of the heated solution gives the phenobarbitone.

Tablets. Shake a portion of the pulverized tablets with 100 ml. of 0.2% sodium hydroxide solution for 10 minutes. Pre-wash the filter paper with water and with 0.2% sodium hydroxide solution. Filter, discarding the first 50 ml. of filtrate. Then treat 25 ml. portions of the filtrate as previously described.

As violuric acid. Evaporate an ethanol solution containing 10-100 mg. of barbitone to dryness. Add 5 ml. of 5% sodium chloride solution and evaporate to dryness. Add 10 ml. of 30% hydrogen peroxide and evaporate to dryness. Repeat with 10 ml. of 10% hydrogen peroxide, then with 10 ml. of 3% hydrogen peroxide.

As a sodium polysulfide reagent, boil 5 grams of sulfur with 100 ml. of 2% sodium hydroxide solution. Filter, and dilute the filtrate to 100 ml. This is stable for only 24 hours. Dissolve the residue of the sample in 3 ml. of water and add 1 ml. of the sodium polysulfide reagent. Heat for 3 minutes and add 1.1 ml. of 1:20 hydrochloric acid. After 1 minute, add 1 ml. of 10% sodium nitrite solution. Heat for 3 minutes and cool. Centrifuge to separate sulfur. Read at 530 $m\mu$. The maximum absorption is reached in 15 minutes.

By cobalt acetate. Tablets. Shake a powdered sample of tablets containing about 80 mg. of barbiturate with 100 ml. of chloroform. Filter. Mix 5 ml. of filtrate with 5 ml. of a 0.125% solution of cobalt acetate in methanol. Add 5 ml. of a 25% solution of isopropylamine in methanol. Read at 560 $m\mu$ against a reagent blank.

*By fluorescence. Amylobarbitone in plasma.*²⁴² Mix 3 ml. of heparinized plasma, containing not less than 0.003 mg. of amylobarbitone, with 3 ml. of water. Shake for 2-3 minutes with 60 ml. of butyl ether and discard the aqueous layer. Adjust 1.36% monopotassium phosphate to pH 8 with 0.4% sodium hydroxide solution. Extract the butyl ether layer with 3, 3, and 3 ml. of the phosphate buffer. Discard the extracts. Shake the butyl ether for 2-3 minutes with 3 ml. of 4% sodium hydroxide solution. Activate the alkaline extract at 265 $m\mu$ and read at 410 $m\mu$.

²⁴² J. E. Swagzdis and T. L. Flanagan, *Anal. Biochem.* 7, 147-51 (1964).

DIPROPHYLLINE AND PHENYLETHYLBARBITURIC ACID

Diprophylline and phenylethylbarbituric acid are determined simultaneously in tablets in alkaline solution.²⁴³

Sample—Dissolve a 0.4-gram sample containing 100-150 mg. of diprophylline and 15-100 mg. of phenylethylbarbituric acid in water and dilute to 200 ml. with water. Mix, and let stand for 1-2 hours. Prepare a buffer at pH 9.5 by diluting 6.18 grams of boric acid and 7.45 grams of potassium chloride to 1 liter. Filter the sample and add 45 ml. of buffer to 5 ml. of the filtrate. Add 34.5 ml. of 0.4% sodium hydroxide solution containing no carbonate. Dilute to 100 ml. with water, mix, and let stand for 15 minutes. Read against the buffer at 240 and 272 m μ . Calculate as follows:

$$\begin{aligned}L_1 &= 0.231 A_{240} - 0.083 A_{272} \\D_1 &= 0.279 A_{272} - 0.0103 A_{240} \\L &= L_1 c/a \\D &= D_1 c/a\end{aligned}$$

in which L and D are contents of diprophylline and phenylethylbarbituric acid, respectively, in grams per tablet; A_{240} and A_{272} are the absorptions at 240 and 272 m μ ; c is the average weight of a tablet in grams; a is the aliquot used, and L_1 and D_1 are the amounts of diprophylline and phenylethylbarbituric acid in the sample in grams.

DILANTIN, PHENYTOIN, DIPHENYLHYDANTOIN

Hydrolysis of the hydantoin ring in strong alkali followed by Hoffman degradation yields an amide, which is steam distilled to a product with a high absorptivity in the ultraviolet. By this method, diphenylhydantoin does not have to be previously separated from other hydantoins, barbiturates, or other drugs.²⁴⁴ The drugs in Table 31 do not interfere. Beer's law is followed for up to 0.02 mg. per ml.

Up to 0.1 mg. per ml. of diphenylhydantoin may be measured in the presence of phenobarbital by extracting the drugs from an acidified sample; separate by partition between chloroform and a borate buffer at pH 8.8, and read. Primidone and methabital interfere.²⁴⁵

After separation by partition between chloroform and the borate

²⁴³ Hanna Bukowska and Jadwiga Gierlowska, *Chem. Anal.* **5**, 309-15 (1960).

²⁴⁴ Jack Wallace, John Biggs and Elmer V. Dahl, *Anal. Chem.* **37**, 410-13 (1965).

²⁴⁵ Ole Svensmark and Poul Kristensen, *J. Lab. Clin. Med.* **61**, 501-507 (1963).

TABLE 31. ULTRAVIOLET ABSORPTION OF BARBITURATES^a

Compound		Absorbance of steam distillate at 257 m μ
Trade name	Chemical name	
Dilantin	5,5-Diphenylhydantoin	1.080
Blank		0.025
Mesantoin	3-Methyl-5-ethyl-5-phenylhydantoin	0.035
Phenurone	Phenacetylurea	0.032
Tridione	3,5,5-Trimethyl-2,4-oxazoladinedione	0.025
Milontin	N-Methyl-2-phenylsuccinamide	0.025
Peganone	3-Ethyl-5-phenylhydantoin	0.025
Phenobarbital	5-Ethyl-5-phenylbarbituric acid	0.025
Veronal	5,5-Diethylbarbituric acid	0.025
Alurate	5-Allyl-5-isopropylbarbituric acid	0.025
Amobarbital	5-Ethyl-5-isoamylbarbituric acid	0.027
Deltoin	1-Methyl-5-phenyl-5-ethylhydantoin	0.025
Aspirin	Acetylsalicylic acid	0.035
	Hydantoin	0.025

^a Each compound was determined from aqueous solution in a concentration of 20 μ g./ml. Each value is the average of three determinations.

buffer, the diphenylhydantoin may be nitrated, reduced with stannous chloride to form an aromatic amine, diazotized, and coupled with N-1-naphthyl ethylenediamine.²⁴⁶

An alternation is to determine by precipitation in 10% sulfuric acid, extraction with ether, and development as the cobalt compound.²⁴⁷ For the separation from phenobarbital for reading in the ultraviolet, see page 526.

Sample—*In the ultraviolet. Serum.* To prepare the borate buffer, dissolve 38 grams of sodium borate in 980 ml. of water. Adjust the pH to 8.8 with 1:10 hydrochloric acid and dilute to 1 liter with water.

Shake a 2-ml. sample, or 1 ml. diluted with water, with 1 ml. of 4% sodium dihydrogen phosphate solution and 5.5 ml. of chloroform. Centrifuge. Add 4 ml. of the filtered chloroform extract, C₁ and 4 ml. of borate buffer, B₁, to a tube. Shake and centrifuge. Transfer the chloroform, C₁, to a tube containing 4 ml. of borate buffer, B₂. Shake and

²⁴⁶ Wesley A. Dill, Anna Kazenko, Loretta M. Wolf and Anthony J. Glazko. *J. Pharmacol. Exptl. Therapy* **118**, 270-4 (1956).

²⁴⁷ Knud Kristensen, *Tidsskr. Farm.* **34**, 97-109 (1960).

centrifuge. Transfer the chloroform, C_1 , to a tube containing 3 ml. of phosphate buffer. Shake and centrifuge. Discard B_2 . Transfer 1 ml. of the phosphate buffer to a semimicro silica cell for reading.

Add 4 ml. of chloroform, C_2 , to B_1 , shake, and centrifuge. Transfer 3 ml. of B_1 to a silica cell for reading and discard C_2 . Read at 235 and 260 $m\mu$ against phosphate buffer. Calculate the diphenylhydantoin concentration from the difference in absorption at the two wave lengths.

By steam distillation. Blood or serum. Shake a 10-ml. oxalated sample vigorously with 2 ml. of 1:23 hydrochloric acid and 100 ml. of chloroform for 5 minutes. After the layers have separated, filter the lower chloroform layer and dilute to 100 ml. Include the correction for the lost chloroform in the calculation. Add 10 ml. of 1.8% sodium hydroxide solution and shake vigorously for 5 minutes. Remove the aqueous layer and centrifuge it for 10 minutes at 2000 rpm.

Steam distil 5 ml. of the supernatant liquid with 100 ml. of 40% sodium hydroxide solution and 0.5 ml. of pure bromine. If the yellow color disappears from the solution after addition of bromine, add additional bromine up to 0.5 ml. The yellow color must exist during the entire period of steam distillation. The distillate should appear at the rate of 5-10 ml. per minute. Collect 50 ml. and read at 257 $m\mu$ against a blank prepared from blood containing no diphenylhydantoin.

If the steam distillate contains less than 0.005 mg. of diphenylhydantoin per ml., concentrate as follows: Extract 50 ml. of distillate by vigorously shaking with 10 ml. of carbon tetrachloride. Evaporate 8 ml. of the carbon tetrachloride phase in a rotary vacuum evaporator, allowing the solution to evaporate by vacuum while the flask rotates at room temperature. Release the vacuum as soon as the carbon tetrachloride is completely removed. Dissolve the residue in 2, 4, or 8 ml. of water, depending on the absorbance at 257 $m\mu$ previously observed for the unextracted distillate.

By nitration, reduction, diazotation and coupling. Plasma, blood or urine. Phenobarbital absent. Add 5 ml. of sample to 0.5 ml. of M phosphate buffer at pH 6.8 and 15 ml. of chloroform. Stopper, and shake mechanically for 15 minutes. Centrifuge to break any emulsions. Transfer 10 ml. of the chloroform layer to a tube containing 6 ml. of 4% sodium hydroxide solution. Shake for 10 minutes to extract the drug into the aqueous phase and centrifuge. Transfer 5 ml. of the sodium hydroxide layer to a tube containing 0.5 ml. of 56% phosphoric acid. The final pH

should be 7 after mixing. Add 6 ml. of chloroform, shake for 10 minutes, and centrifuge.

Evaporate 5 ml. of the final chloroform extract to dryness by heating for 30 minutes at 100°. The tube should be completely surrounded by the heating medium and no liquid residue should remain. To the residue, add 0.25 ml. of 0.1% potassium nitrate solution in concentrated sulfuric acid as a nitrating agent. Rotate the tube by hand. After 30 minutes, add 0.5 ml. of 1.6% stannous chloride solution in 3:1 hydrochloric acid and let stand at room temperature for at least 10 minutes. For the diazotization, add 0.5 ml. of 1% sodium nitrite solution. Mix, and let stand for 5 minutes. Add 0.5 ml. of 4% ammonium sulfamate solution to remove the yellow color. Add 4 ml. of water, 1 ml. of a 1:1 mixture of *n*-butanol and isopropanol, and 0.5 ml. of 0.2% *N*-1-naphthylethylenediamine dihydrochloride solution. Mix thoroughly, let stand for 30 minutes at room temperature, and read at 550 m μ against a reagent blank.

Tissues. Phenobarbital absent. To a 0.5-2-gram sample, add water to give a final volume which is 20-fold greater than the weight of the tissue in grams. Homogenize in a blender for 5 minutes. Take a 5-ml. portion of the homogenate and follow the procedure for plasma, blood, and urine samples by nitration, reduction, diazotation, and coupling, starting at the beginning.

Plasma, blood, or urine. In the presence of phenobarbital. Follow the procedure under "By nitration, reduction, diazotation, and coupling. Phenobarbital absent," starting at the beginning and ending with "Centrifuge to break any emulsions."

Place 10 ml. of the chloroform layer from the procedure in tube A and shake for 10 minutes with 10 ml. of 0.2 *M* borate buffer at pH 9. Transfer the chloroform layer to tube B and shake again with an equal volume of fresh borate buffer. Transfer the chloroform phase to tube C. Add fresh chloroform to tube A containing the original borate buffer and shake. Transfer this chloroform layer to tube B and shake again with the borate buffer in that tube. Add this chloroform extract to the chloroform extract in tube C. Add 6 ml. of 4% sodium hydroxide solution to tube C and shake for 10 minutes. Transfer 5 ml. of the sodium hydroxide layer to another tube and neutralize with 0.5 ml. of 56% phosphoric acid to a final pH of 7. Add 6 ml. of chloroform and shake for 10 minutes. Develop a 5-ml. portion of the chloroform extract following

the procedure for blood, plasma, or urine, starting at "Evaporate 5 ml. of the final chloroform extract . . ."

By cobalt acetate. Mix 2 ml. of sample solution with 5 ml. of 1:17 sulfuric acid. Shake with 100 ml. of ether. Separate the solvent layer and evaporate in a stream of cold air. Take up the residue in 10 ml. of chloroform. Add 5 ml. of a 1% solution of cobalt acetate and 5 ml. of isopropylamine. Dilute to 25 ml. with chloroform and read at 570 $m\mu$. The color is stable for over 2 hours.

METHAQUALONE, 2-METHYL-3-*o*-TOLYL-4(3H)-QUINAZOLONE

Methaqualone is described as a nonbarbiturate hypnotic. It is determined by diazotized sulfanilic acid or 4-nitrobenzenediazonium chloride.²⁴⁸ Beer's law holds to about 0.1 mg. per ml.

Procedure—*By diazotized sulfanilic acid.* Prepare diazotized sulfanilic acid by mixing 0.5 ml. of 1% sulfanilic acid in 1:10 hydrochloric acid with 0.25 ml. of 1% sodium nitrite solution. Heat methaqualone in 1:10 hydrochloric acid for 1 hour at 100° to hydrolyze. Add the reagent to 5 ml. of the hydrolyzed solution and heat at 70° for 30 minutes. Cool, and read at 400 $m\mu$.

By 4-nitrobenzenediazonium chloride. Prepare the reagent from 0.2 gram of 4-nitroaniline in 3 ml. of concentrated hydrochloric acid and 5 ml. of glacial acetic acid by cooling to 0°. Add 1 ml. of 15% solution of sodium nitrite and dilute to 50 ml. with glacial acetic acid. Adjust the pH of the sample to 2, and to an appropriate volume, add 1 ml. of the reagent. Heat to 80° for 50 minutes and cool. Read at 430 $m\mu$.

ETHOSUXIMIDE, 2-METHYL-2-METHYLSUCCINIMIDE

Ethosuximide is read in the ultraviolet after extraction.²⁴⁹

Procedure—*Serum.* Extract from 0.5 ml. of serum by shaking with 10 ml. of chloroform for 3 minutes. After the layers separate, filter the chloroform. Shake 4 ml. of the extract with 5 ml. of 0.64% solution of

²⁴⁸ Masaru Nakano, *Arch. Pract. Pharm., Japan* 22, 158-60 (1962); *ibid.* 24, 49-52 (1964).

²⁴⁹ S. E. Hansen, *Acta Pharm. Tox., Kbh.* 20, 286-90 (1963).

trisodium phosphate dodecahydrate. Centrifuge, and read the aqueous layer at 240 $m\mu$ and 218 $m\mu$ against the phosphate solution as a blank. Add 0.025 ml. of 1:1 hydrochloric acid to the sample and blank, and read again. Determine by the difference in extinction at 218 $m\mu$ less the difference in the reagent blank. Any significant change in the reading at 240 $m\mu$ indicates the presence of barbiturates that must be corrected for.

5-(*p*-HYDROXYPHENYL)-5-PHENYLHYDANTOIN

Potassium ferricyanide is a reagent for 5-(*p*-hydroxyphenyl)-5-phenylhydantoin.²⁵⁰

Sample—Urine. To prepare the acetate buffer solution, dissolve 41.02 grams of anhydrous sodium acetate and 10 ml. of acetic acid in water and dilute to 500 ml. To prepare the borate buffer, dissolve 25 grams of boric acid in water, adjust the pH to 10 with sodium hydroxide, and dilute to 1 liter.

Autoclave a 5-ml. sample with 5 ml. of concentrated hydrochloric acid at 15 pounds per square inch for 30 minutes. Cool, and place in ice for 2-3 minutes. Slowly add 6 ml. of 40% sodium hydroxide solution. Warm to room temperature and dilute to 50 ml. with water. To a 10-ml. aliquot, add 5 ml. of acetate buffer solution and 20 ml. of 1,2-dichloroethane. Shake for 10 minutes and centrifuge. Shake 15 ml. of the solvent layer with 6 ml. of borate buffer solution for 10 minutes and centrifuge. To 4 ml. of the aqueous layer, add 1 ml. of 0.25% aminophenazone solution and 1 ml. of 0.8% potassium ferricyanide solution. Read at 510 $m\mu$ against a reagent blank within 1-2 minutes.

HEXAMETHYLENEDIAMINE

Hexamethylenediamine is developed with sodium nitroprusside.²⁵¹ Results compare favorably with determination by chloroplatinate.

Procedure—Mix 10 ml. of sample, 0.5 ml. of methylacetate and 0.5% of 0.5% sodium nitroprusside solution. After a few minutes, read at 530 or 550 $m\mu$.

²⁵⁰ T. Panalaks, *Clin. Chim. Acta* **8**, 968-70 (1963).

²⁵¹ Rosarina Carpignano and Giovanni Musso, *Ann. Chim. (Rome)* **44**, S21-6 (1954).

UROTROPIN, HEXAMETHYLENETETRAMINE, METHENAMINE,
AND METHENAMINE MANDELATE

After hydrolysis of a sample to remove interference from formaldehyde, methanamine and methenamine mandelate are nesslerized²⁵² (cf. Vol. IV, p. 56). The final product is stable for 1 hour. Constant final pH values must be maintained. Amino nitrogen is not affected by hydrolysis. Beer's law is followed for 0.0027-0.027 mg. of methenamine mandelate per ml., equivalent to 0.52-5.23 micrograms of nitrogen per ml. An alternate is to convert back to formaldehyde (Vol. IV, pp. 56-7). The formaldehyde is then determined by chromotropic acid²⁵³ (Vol. IIIA, p. 252).

Procedure—*Bacterial sensitivity disks.* To obtain complete elution from the sensitivity disks, shake the paper with 1:3500 sulfuric acid for a few minutes. Dilute the sample with 1:3500 sulfuric acid so that the concentration of methenamine is 0.01-0.1 mg. per ml. and the concentration of methenamine mandelate is 0.02-0.2 mg. per ml. Concentrate a 3-ml. aliquot to 0.5 ml. by boiling.

Add 5 ml. of water and reconcentrate to 0.5 ml. Repeat with two 5-ml. portions of water. Cool to room temperature and dilute to 20 ml. with water. Add 1 ml. of Nessler's reagent and dilute to 25 ml. with water. Read against a reagent blank at 465 m μ after 10 minutes. Dilute a 3-ml. solution of ammonium sulfate containing 0.025 mg. of nitrogen per ml. to 20 ml. with water. Follow the above procedure, starting at "Add 1 ml. of Nessler's reagent . . ."

Calculate methenamine as follows:

$$\text{Concentration in micrograms per ml.} = \frac{A \times 2.502}{a}$$

in which A is the absorbance of the sample at 465 m μ ; a is the average absorbance of ammonium sulfate standards per microgram of nitrogen per ml.; and 2.502 is the conversion factor for nitrogen to methenamine.

Calculate methenamine mandelate by the same formula, substituting 5.217 for 2.502.

²⁵² Bertram W. Griffiths and James E. Logan, *Anal. Chem.* 31, 1882-4 (1959).

²⁵³ Tadashi Shiota, Mashahito Kotakemori and Masakazu Harima, *Eisei Kagaku* 6, 107-10 (1958).

PIPECOLIC ACID, PIPECOLINIC ACID, HEXAHYDROPICOLENIC ACID
PIPERIDINE-2-CARBOXYLIC ACID

In an acid medium, pipercolic acid forms a color with ninhydrin.²⁵⁴ The method is sensitive to water concentration, to the type of acid used, and to the age of the ninhydrin. For example, the sample dissolved in 0.1 ml. of water produces a color approximately half that of the color produced by the recommended procedure. Lysine interferes, since it forms a small amount of pipercolic acid upon nitrous acid treatment. Separation on ion-exchange resins removes interferences from salts and α -amino acids. Since sugars and nonionic materials prevent this separation, they are removed first by retaining the ionic material on the resin. Heavy metals that also interfere are removed in this step.

In the separation of pipercolic acid from α -amino acids and salts, the dimensions of the glass column are important. In the separation of non-ionic substances, the dimensions are not important. The acid groups on the resin must be neutralized to prevent retention of the pipercolic acid by the resin. Excessive washings of the resin results in subsequent retention of amino acids. Sample solutions of low pH act in the same manner.

Pipercolic acid, 4-hydroxypipercolic acid, and 4-aminopipercolic acid are isolated from plant extracts by adsorption on Dowex 50-X4.²⁵⁵ They are separated by paper chromatography by 3:1 propanol-1:4 ammonium hydroxide. The fractions are determined by ninhydrin. *Cis*- and *trans*-isomers of 4-hydroxypipercolic acid are separable by paper chromatography, using 18:2:5 butanol-acetic acid-water.

Sample—*Removal of sugars, nonionic material, heavy metals, α -amino acids, and salts.* Prepare glass tubes 0.9×50 cm. with an opening of 3-4 mm. at the lower end. For the separation of sugars from amino acids, treat a 200-400 mesh ion-exchange resin, Dowex L-X2, with an excess of carbonate-free 12% sodium hydroxide solution overnight and wash away the base with water. Insufficient removal of excess base from the resin results in incomplete separation of pipercolic acid.

For the separation of pipercolic acid from salts and α -amino acids, treat a Dowex 50-X12 resin with an excess of 1:3 hydrochloric acid at 100° for 24 hours. Remove the excess acid and neutralize the resin with excess 12% sodium hydroxide solution at 100° for 24 hours. Wash the

²⁵⁴ Richard S. Schweet, *J. Biol. Chem.* **208**, 603-13 (1954); O. O. Silverstein, R. M. Adjarian and J. F. Thompson, *Anal. Chem.* **28**, 855-7 (1956).

²⁵⁵ W. Schenk, *Biochem. Z.* **336**, 557-63 (1963).

resin with water to remove excess base until the pH of the wash water reaches 10 ± 0.2 . The resin is now in the sodium form.

Grind moist samples with sufficient 95% ethanol solution so that the resultant alcohol concentration is not less than 75%. Separate the liquid from the residue and repeat the extraction with 75% ethanol solution as many times as necessary to extract amino acids. Treat dry samples similarly, starting with 75% ethanol solution. Samples containing carbonates should be acidified before application to resin columns, since the carbon dioxide released in the resin disrupts the column.

To separate the nonionic substances from ionic substances, pass the extract of the sample through a column of the prepared Dowex L-X2 resin. To make sure that all amino acids are retained on the column, test the effluent with ninhydrin. Wash the resin with deionized water until tests for sugar are negative, and discard the washings.

Elute the amino acids and pipecolic acid from the resin with 1:10 hydrochloric acid until the effluent is acid. Dry the eluate at room temperature and dissolve in water. Adjust the pH to 9.5-10.5 and dilute so that 1 ml. contains 0.001-0.02 mg. of pipecolic acid. Pour the prepared Dowex 50-X12 resin for the separation of pipecolic acid from α -amino acids and salts, as a thick slurry, into the glass column, using a filter paper disk at the bottom to retain it. The height of the settled resin should be 30 cm. Carefully place a 1-ml. aliquot of extract free from nonionic substances at the top of the resin. Wash the sides of the column with two successive 1-ml. portions of water, allowing the water to pass into the resin each time. Add 13 ml. of water. Collect the effluent containing α -amino acid and salts. Then add 10 ml. of water and collect the pipecolic acid. Dry the fraction and develop pipecolic acid with ninhydrin.

Removal of hydrochloric acid. To remove small amounts of hydrochloric acid from pipecolic acid samples, place an aliquot of the eluate in the test tube to be used for color development and place in a vacuum desiccator over sodium hydroxide. Dry with a vacuum pump and a dry ice trap. Add 0.2 ml. of water and dry again. Repeat with an additional 0.2 ml. of water. Remove from the desiccator, add 0.05 ml. of water, and develop with ninhydrin.

Procedure—By ninhydrin. Evaporate a sample containing 0.001-0.02 mg. of pipecolic acid to dryness in flowing air at room temperature. Add 1 ml. of 4% ninhydrin solution in a mixture of 9 parts of 1-butanol and 1 part of 85% phosphoric acid. Stopper, shake, and heat at 100° for 15 minutes. Cool rapidly and dilute to 5-10 ml. with ethyl acetate. Keep

the tube out of intense light during and after heating to prevent fading. Read at 575 $m\mu$ within 1 hour. If proline is present, read at 510 $m\mu$ and calculate pipecolic acid as follows:

$$D_{575} = APi + BPr$$

$$D_{510} = CPi + DPr$$

$$D_{575} + D_{510} = \text{density measurements at 575 and 510 } m\mu, \text{ respectively}$$

where Pi and Pr are micrograms of pipecolic acid and of proline; A is the density from 1 microgram of pipecolic acid at 575 $m\mu$; B is the density from 1 microgram of proline at 575 $m\mu$; C is the density from 1 microgram of pipecolic acid at 510 $m\mu$; and D is the density from 1 microgram of proline at 510 $m\mu$. To determine concentrations of pipecolic acid up to 0.1 mg., dilute the ethyl acetate further. Larger quantities of pipecolic acid produce insoluble precipitates.

MELAMINE, 2,4,6-TRIAMINO-S-TRIAZINE, CYANUROTRIAMIDE, AND RELATED COMPOUNDS

Melamine resin is extracted and hydrolyzed in hydrochloric acid to the melaminum ion, which is read at 235 $m\mu$.²⁵⁶ To correct for any ammeline resulting from hydrolysis of melamine, readings are also taken at 237 $m\mu$. The absorbance at 260 $m\mu$ is used for the background absorption and is subtracted from the reading at 235 $m\mu$. The method is sensitive to 0.004 mg. of melamine in the absence of interferences.²⁵⁷ Lard samples are extracted with dilute hydrochloric acid and isooctane. Commercial antioxidants in lard interfere. Melamine and melamine resin, and trimethylolmelamine are distinguished from each other by their spectra when present in large amounts, but they cannot be distinguished in low concentrations such as are encountered in lard samples. A correction is applied for the absorbing material, which is also extracted.

Melamine, ammeline, ammelide, and cyanuric acid are read in hydrochloric acid and borax.²⁵⁸ The first two are read in 0.05% solution in 1:110 hydrochloric acid solution at 215 and 230 $m\mu$ and solved by simultaneous equations. Similarly, in 0.05% solution in 1.7% sodium tetraborate at 216 and 231.5 $m\mu$, the latter two are solved by simultaneous equations.

²⁵⁶ R. C. Hirt, F. T. King and R. G. Schmitt, *Anal. Chem.* **26**, 1273-4 (1954).

²⁵⁷ R. C. Hirt, W. R. Doughman, R. G. Schmitt and S. T. Moore, *J. Agr. Food Chem.* **3**, 1044-6 (1955).

²⁵⁸ E. N. Boitsov and A. I. Finkel'shtein, *Zh. Analit. Khim.* **17**, 748-50 (1962); A. I. Finkel'shtein, E. N. Boitsov, and Yu. I. Mushkin, *Zavod. Lab.* **30**, 44-5 (1964).

Melamine, ammeline, ammelide, and cyanuric acid are separable by ion-exchange chromatography.²⁵⁹ Thus, Amberlite IR-120 takes up the first three but not cyanuric acid. Thereafter, ammelide is eluted with 1:120, ammeline with 1:20, and melamine with 1:5 hydrochloric acid. They are then each read in the ultraviolet. Similar techniques are applicable to melam, melem, cyanomelamine, guanylmelamine, and cyameluric acid.

Procedure—Paper. Cut a 1-2-gram sample into small pieces and weigh. Reflux with 100 ml. of 1:110 hydrochloric acid for 1 hour in a flask with ground glass stopper and joint connections. Filter, and read against 1:110 hydrochloric acid at 260 $m\mu$, 235 $m\mu$, and 237 $m\mu$. Calculate melamine as follows:

$$\text{per cent of melamine} = \frac{(A_{237} - A_{260})(f)(V)}{(a_{237})(b)(W)}$$

in which A is the observed absorbance at the designated wave length; f is the dilution factor; V is the volume of hydrochloric acid in ml.; a is the absorptivity which at 237 $m\mu$ is 79.0; b is the cell light path length in mm.; and W is the sample weight in grams.

Lard. Dissolve a 15-gram sample in 100 ml. of isooctane, add 10 ml. of 1:110 hydrochloric acid, and shake well. Let stand for 20 minutes, remove the bottom acid layer, and re-extract the remaining layer with another 10-ml. portion of acid. Combine the extracts. Read against 1:110 hydrochloric acid by scanning from 270 $m\mu$ to the lower wave length limit of the instrument. Record the readings at 235 and 260 $m\mu$ for sample and blank, and calculate the concentration of melamine and/or melamine resin as follows:

$$c_m = \frac{A_{235} - (R_{av.} \times A_{260})}{(b)(a_{235})}$$

in which c_m is the concentration of melamine in grams per 100 ml.; A_{235} and A_{260} are the observed absorbances at the wave lengths indicated; b is the cell light path in mm.; a_{235} is the absorptivity of melamine at 235 $m\mu$ (81.0); and $R_{av.}$ is the average of the ratio of observed absorbances at 235 and 260 $m\mu$ of the extracts of the blank samples.

The concentration of melamine in the sample is as follows:

$$C_m = \frac{c_m V}{W}$$

²⁵⁹ Mayavoshi Takimoto, *Kogyo Kagaku Zasshi* 64, 1234-8, 1452-60 (1961).

in which C_m is the concentration of melamine in the sample; V is the volume of the extracting hydrochloric acid in ml.; and W is the weight of the sample in grams.

Melamine and its hydrolysis products, ammeline, ammelide, and cyanuric acid. Dissolve a 0.05-gram sample in 100 ml. of 2% borax solution. Dilute 1 ml. to 100 ml. with 1:100 hydrochloric acid. Dilute 1 ml. to 100 ml. with 0.4% borax solution. Read the acid solution at 215 and 230 $m\mu$, the alkaline solution at 216 and 231.5 $m\mu$. Solve by equations.

GLYCAMIDE, GLYCARBYLAMIDE, 4,5-IMIDRAZOLEDICARBOXAMIDE

A method for the analysis of glycarbylamide involves extraction from feed samples with dimethylformamide, purification of the extract on alumina and Amberlite IRA-400, bromine oxidation of traces of interferences not removed by chromatography, mercuric oxide adsorption of glycarbylamide from part of the purified solution, and differential measurement at 283 $m\mu$ in alkaline solution.²⁶⁰

Procedure—Feeds. To prepare the aluminum oxide, shake 10 grams vigorously with 100 ml. of water for at least 2 minutes. Let settle, and decant. The pH determined electrometrically should be 10-10.5. As a column for aluminum oxide, use a 50-cm. \times 22-mm. glass tube constricted at one end. Place a glass wool plug at the constricted end and pack in 15 grams of aluminum oxide by gentle tapping on the side of the tube. Wash the column with 25 ml. of dimethylformamide and allow the solvent to drain to 1-2 cm. above the resin level before adding the sample.

To prepare the Amberlite IRA-400, wash the resin in a glass column with 250-ml. portions of the following reagents in the order listed: water, 10% sodium hydroxide solution, water, 1:90 hydrochloric acid, water, and 10% sodium hydroxide solution. Wash with water until the eluate is neutral and store under water in polyethylene bottles.

A glass tube, 24 cm. \times 9 mm., serves as a column for the Amberlite IRA-400 resin. Seal the upper end to a reservoir of 8-cm. \times 5-cm. tubing. Constrict the lower end. Attach the lower end to polyethylene tubing. Add a glass wool plug to the constricted end and add a water slurry of the Amberlite-400 resin to a level of 4 inches above the plug after the

²⁶⁰ C. R. Czalkowski, *J. Assoc. Offic. Agr. Chemists* **43**, 284-6 (1960).

water has been drained from the resin. Backwash the resin into the reservoir with water and let it settle into the column with no flow through the column. Let the water drain to 1 cm. above the top of the resin.

Add 200 ml. of dimethylformamide to a weighed ground sample not greater than 25 grams, containing up to 1.2 mg. of glycarbylamide. Stir vigorously for 1 hour. Centrifuge the suspension for 3-5 minutes or filter by suction with the aid of 5-10 grams of Supercel or other filter aid. Add 100 ml. of the filtrate to the aluminum oxide column and let the column drain by gravity. Wash the column with two 15-ml. portions of dimethylformamide followed by two 25-ml. portions of anhydrous methanol, discarding the washings.

Elute the column with four 50-ml. portions of water. Pass the entire eluate through the Amberlite IRA-400 column at a rate of 2-3 ml. per minute. Do not permit the liquid level to drain into the resin bed at any time. Wash the column with one 10-ml. portion and two 20-ml. portions of water. Drain at a flow rate of about 3 ml. per minute, discarding the eluate and washings.

Elute the Amberlite column with 15-ml. portions of 1:9 hydrochloric acid at a rate of 1-2 ml. per minute. Discard the first 3 ml. of eluate and collect the next 50 ml.

As a bromine oxidizing solution, dissolve 1.5 grams of potassium bromate and 7.5 grams of potassium bromide in water, and dilute to 250 ml. To prepare the alkali solution, dissolve 20 grams of sodium metaborate and 45 grams of sodium hydroxide in water, and dilute to 200 ml.

To 25 ml. of the eluate, add 1 ml. of the bromine solution. Mix, and let stand for 3 minutes to oxidize any remaining traces of interferences. Add 1 ml. of 2% sodium bisulfite solution and let stand for 3 minutes. Add 5 ml. of the alkaline solution and mix carefully. Remove 10 ml. of the solution and label it A. To the remaining solution, add 1 gram of red mercuric oxide, stopper, and shake vigorously for 10 minutes to adsorb glycarbylamide. Centrifuge. Remove 10 ml. of the supernatant liquid and label it R. Add 1 ml. of 1% potassium cyanide solution in 4% sodium hydroxide solution to both A and R, and mix. Read tube A against R at 283 $m\mu$ within 3 minutes. Do not expose tube R unnecessarily to light.

$$\% \text{ glycarbylamide in sample} = \frac{1.28 AC}{A_s W}$$

in which A is the absorbance of the sample; A_s is the absorbance of the standard; C is the mg. of glycarbylamide in the final aliquot of standard; and W is the weight of the original sample in grams.

VITAMIN B₂, RIBOFLAVIN, 6,7-DIMETHYL-9-(D-1'-RIBETYL)
ISOALLOXAZINE

The most common method of measuring riboflavin is by its yellow-green fluorescence as lumiflavin (Vol. IV, pp. 296-308). In cereal samples, addition of methanol precipitates colloidal and dispersed materials, and allows filtration.²⁶¹ The interference caused by the fluorescence of highly colored cereal products can be greatly diminished through oxidation by an increase in permanganate concentration and in its reaction time.²⁶² Anthranilic acid and its conjugates, folic acid, pyridoxine, and riboflavin metabolites interfere more or less, but potassium permanganate is the best reagent to reduce their interference.²⁶³

Food is extracted with 1:110 hydrochloric acid at 121° after a short fermentation with diastase. Thereafter, it is read fluorimetrically.²⁶⁴ This applies to grains, flours, fish meal, etc.

For determination in feed, thiamine is oxidized with ferricyanide and riboflavin is read directly.²⁶⁵ For riboflavin in milk, hydrolyze by acidifying with sulfuric acid, and heat at 100° for 45 minutes. Buffer by addition of sodium acetate and oxidize with potassium permanganate. Precipitate the proteins with sodium metaphosphate and acetic acid. Incubate at 37° for 24 hours and filter. Read fluorometrically.²⁶⁶ Riboflavin is also read at 445 m μ in pharmaceutical products after addition of dithionate.²⁶⁷ The method is more flexible than fluorometric reading and applicable over a wider range of pH. Sensitivity is less than for the fluorometric method. Artificial coloring is often so eliminated. Adsorbed riboflavin may be extracted with 30:10:10:1 methanol-pyridine-water-acetic acid.²⁶⁸

Riboflavin in citrated blood and tissue extracts is determined by heating at 80° for 15 minutes. Thereafter, the solution is mixed with 10%

²⁶¹ R. H. Anderson and D. F. Moran, *Cereal Chem.* 39, 463-8 (1962).

²⁶² W. G. Bechtel, *Cereal Sci. Today* 7, 198-201 (1962).

²⁶³ Isao Ishiguro, Kiheiiji Tanaka and Osamu Kinoshita, *Vitamins (Japan)* 8, 452-7 (1955).

²⁶⁴ B. Gassmann and H. Plessing, *Z. Lebensm.-Untersuch. u.-Forsch.* 109, 135-45 (1959).

²⁶⁵ L. Stefanovičs, *Latvijas PSK Zinātņu Akad. Vestis* 1954, No. 8, 75-80.

²⁶⁶ Aleksander Lempka, Anna Muzynska and Jacek Koziol, *Pyżemysl Spożywczy* 11, 298-302 (1957).

²⁶⁷ L. Brealey and D. A. Elvidge, *J. Pharm. and Pharmacol.* 8, 885-94 (1956).

²⁶⁸ M. J. Deutsch, H. C. Pillsbury, S. S. Schiaffino and H. W. Loy, *J. Assoc. Off. Agr. Chemists*, 43, 42-3 (1960).

trichloroacetic acid and centrifuged. Riboflavin in the supernatant layer is read fluorescently.²⁶⁹ Somewhat similarly, urine is diluted with 1.5 volumes of water and 2.5 volumes of 20% trichloroacetic acid, and heated at 100° for 5 minutes. A chloroform extraction is made and the extract discarded. Thereafter, the riboflavin is read at pH 13-14 as lumiflavin.²⁷⁰

Riboflavin, flavin, mononucleotide, lumiflavin, and lumichrome are separable by paper chromatography by 5% disodium phosphate solutions.²⁷¹ The riboflavin spot is eluted by 50% methanol for reading as lumichrome before and after addition of 10 mg. of sodium thiosulfate. The others are similarly extracted with 10% acetic acid and read before and after addition of thiosulfate. Flavines are present in the free form as mononucleotide and as the adenosine nucleotide.²⁷² Background interference is removed from tissue samples by treatment with 1% permanganate and hydrogen peroxide. Total fluorescence is read at 550 mμ. The loss in fluorescence by extraction with benzyl alcohol for 30 minutes at room temperature gives acriflavine. The nucleotide is given by the increase in fluorescence by immersion in 1% takadiastase for 20 minutes at 38°. For riboflavin and thiamine simultaneously in the ultraviolet, see page 435.

Procedure—Urine.²⁷³ Collect a sample with 20 ml. of 1:9 sulfuric acid under refrigeration. In the dark, heat a 50-ml. portion at 100° for 30 minutes. Cool to 45° and adjust the pH to 4.5-5.4 with sodium acetate solution. Add 2 grams of diastase. Incubate for 45 minutes at 45°. Cool, dilute to 100 ml. with water, and filter. Extract an appropriate aliquot of the filtrate with 1.5 parts of fluorescence-free chloroform. Separate the phases by centrifuging.

Pipet 10-ml. portions of the aqueous phase into shallow evaporating dishes. To one-half of the samples, add 2 ml. of a standard solution containing 0.001 mg. of riboflavin per ml. To the other half, add 2 ml. of

²⁶⁹ Kunio Yagi, Saburo Kikuchi and Toru Kariya, *Vitamins* (Japan) **8**, 450-1 (1955).

²⁷⁰ P. D. Sharshov, *Lab. Delo* **8**, No. 6, 37-42 (1962).

²⁷¹ Fumihiko Murai, Takeo Okubo, and Chang Kuo Shein, *Vitamin* **16**, 392-5 (1959); P. Cerletti and P. L. Ipata, *Biochem. J.* **75**, 115-19 (1960); B. Gassmann, *Nahrung* **4**, No. 2, 98-115, 140-a, (1960); J. Travis and A. D. Robinson, *Canad. J. Biochem. Physiol.* **40**, 1251-2 (1962).

²⁷² Chentung Chen and Kazuo Yamauchi, *J. Vitaminol.* (Kyoto) **7**, 163-81 (1961).

²⁷³ B. Gassmann, *Ernährungsforschung* **3**, 400-13 (1958); P. D. Sharshov and S. M. Kirov, *Voprosy Med. Khim.* **4**, 155-60 (1958); P. D. Sharshov, *Lab. Delo* **8**, No. 6, 37-42 (1962).

water. Mix vigorously and add 2 ml. of 28% sodium hydroxide solution immediately to each. Start photolysis for 30-45 minutes at 20°, using a 500-watt lamp with its filament at a distance of 30 cm. from the bottom of the evaporating dish. After photolysis, add 2 ml. of glacial acetic acid and 0.3 ml. of 3% potassium permanganate solution to each dish. After 1 minute, add 0.3 ml. of 1.7% hydrogen peroxide solution. Take up each in 5 ml. of water and add 30 ml. of chloroform. Centrifuge and aspirate the aqueous layer. Dry the chloroform layer with anhydrous sodium sulfate, centrifuge, and read fluorometrically.

*Foodstuffs.*²⁷⁴ *Macerate* an 80-gram sample with 200 ml. of 1:350 sulfuric acid. Heat at the boiling point, stirring constantly for 45 minutes. Cool, adjust the pH to 4.5 with 2% sodium acetate solution containing 2.4 grams of takadiastase. Incubate overnight at 37° and cool. Dilute to 600 ml. and centrifuge. Adsorb riboflavin on fuller's earth and elute with two 45-ml. portions of 20% pyridine solution in 2% acetic acid. Add 5 ml. of 4% potassium permanganate solution, stir, and add enough 3% hydrogen peroxide solution to decolorize. Dilute to 100 ml. with 20% pyridine in 2% acetic acid solution. Filter, and determine the fluorescence of riboflavin.

*Animal feedstuffs.*²⁷⁵ Heat a 20-gram sample with 300 ml. of water to incipient boiling, stirring occasionally. Cool slowly to 60° and add 2 grams of Clarase. After 10-12 hours, dilute to 500 ml. and filter, discarding the first 30 ml. Read the fluorescence, using an internal standard against a blank of the test solution to which 20 mg. of sodium hydrosulfite has been added.

*Tissue.*²⁷⁶ Cut up 0.1-0.5 gram, wet weight, and digest with water at 80° for 3-5 minutes. Homogenize, and let stand at 80° for 15 minutes. Cool, dilute to a known volume, and centrifuge.

Dilute 1 ml. of the supernatant layer with 1 ml. of water and 2 ml. of 4% sodium hydroxide solution. Place this and a standard tube containing 0.0001 mg. of riboflavin in a closed box. Irradiate with a Mazda FL 30D lamp, radiation below 530 m μ , for 30-60 minutes to convert the riboflavin

²⁷⁴ Brajadulal Mukherjee and K. Ahmad, *Pakistan J. Biol. Agr. Sci.* **1**, 47-51 (1957).

²⁷⁵ R. DeGori, P. Cantagalli and F. Grandi, *Boll. Lab. Chim. Provinciali* **15**, 360-6 (1964).

²⁷⁶ Kunio Yagi, *J. Biochem. (Japan)* **43**, 635-44 (1956); *Bull. soc. chim. France*, 1543-50 (1957).

to lumiflavin. Add 0.2 ml. of glacial acetic acid, cool, and extract with 6 ml. of chloroform saturated with water. Read the fluorescence as compared to a blank.

*Milk and milk products.*²⁷⁷ Dilute a 2-ml. milk sample with 10 ml. of water and dilute other milk-product samples to a similar concentration. Add 4 ml. of 10% trichloroacetic acid solution, and after 30 minutes, centrifuge. To a 2-ml. portion of the supernatant liquid, add 2 ml. of 8% sodium hydroxide solution. Photolyze for 1 hour with three 100-watt lamps of 85 volts or higher, at a distance of 25 cm., at 20°. Shake with 0.4 ml. of glacial acetic acid and 12 ml. of chloroform vigorously for 2 minutes. Centrifuge, and read the fluorescence of the chloroform layer by a photofluorometer.

Bread. Dry slices in a warm, dark room with air circulating until the sample is in equilibrium with the moisture of the air. Weigh the dried sample to determine moisture loss and grind the dried sample to pass through a 20-mesh sieve. Mix thoroughly. Weigh a sample according to the following schedule: For samples containing 0-0.8 mg./lb. of riboflavin, weigh 5 grams; for 0.8-2 mg./lb., weigh 4 grams; and for 2-4 mg./lb., weigh 2 grams. To the sample, add 75 ml. of 1:350 sulfuric acid. Mix, and autoclave at 15 lb./sq. in. for 30 minutes, or heat at 100° for 30 minutes, shaking the solution every 5 minutes. Let cool to room temperature. Add 5 ml. of 34% sodium acetate solution, mix, and let stand for at least 1 hour. The pH of the solution is approximately 4.5. Dilute to 100 ml. and filter through medium-fast paper, discarding the first 10-15 ml. In two tubes of 1-in. diameter containing separate stirring rods marked A and B, carry out the oxidation as shown in Table 32.

If a precipitate forms upon oxidation, a longer period should be allowed. If the blank reading is 20% or more of the sample reading by the low-blank procedure, the high-blank procedure should be used. Stir the samples after each addition of permanganate. Shake after adding peroxide until foaming is negligible, to prevent formation of air bubbles in the cuvetts. Addition of up to 0.2 ml. of caprylic alcohol controls foaming.

²⁷⁷ Rijnro Sasaki, Tomokichi Tsugo, Masao Fujimaki and Satoru Oka, *Nippon Nogei Kagaku Kaishi* **28**, 20-4 (1954); cf. Aleksander Lempka, Anna Muszynska, and Jacek Kozioł, *Przemysł Spożywczy* **11**, 298-302 (1957); L. Sorman, *Chem. Zvesti* **17**, 798-82 (1963).

TABLE 32. TREATMENT OF SAMPLES FOR RIBOFLAVIN

	<i>Low Blank Material</i>		<i>High Blank Material</i>	
	A	B	A	B
Sample solution	10 ml.	10 ml.	10 ml.	10 ml.
Standard solution (0.001 mg. of riboflavin per ml.)	1 ml.	...	1 ml.	...
Water	1 ml.	2 ml.	...	1 ml.
4% Potassium permanganate solution	0.5 ml.	0.5 ml.	1 ml.	1 ml.
Time	2 min.	2 min.	4 min.	4 min.
3% Hydrogen peroxide	0.5 ml.	0.5 ml.	1 ml.	1 ml.

Determine the fluorescence of tubes A and B with no more than 10 seconds exposure in the fluorometer. To solution B in the cuvet, add 20 mg. of sodium hydrosulfite, stir, and determine the blank fluorescence, C. Do not use reading C after colloidal sulfur begins to form.

$$\text{Riboflavin mg./lb.} = \frac{B - C}{A - B} \times \frac{10}{S} \times 0.454$$

Whole grain products, grits, meal, flaked and puffed cereals, farina. Grind a large sample and pass through a 20-mesh sieve. Mix milled material thoroughly. Follow the procedure under "Bread," starting with "Weigh a sample according to the following schedule . . ."

High potency toasted cereal flakes. Mix a 2-gram sample containing a minimum of 1.2 mg. of riboflavin per 28 grams at low speed in a blender for 5 minutes with 200 ml. of 1:9 acetic acid. Mix for 10 minutes at the highest speed possible without loss of sample by splashing. During extraction, protect the sample from light.

As an alternative to blending, shake a 2-gram finely ground sample for 15 minutes on a mechanical shaker with 200 ml. of 1:9 acetic acid. Allow the mixture to settle for 2 minutes. Mix a 25-ml. portion of the supernatant liquid with 50 ml. of methanol to precipitate colloidal and dispersed materials. Filter, and read the fluorescence of a 15-ml. portion of the filtrate after the galvanometer of the fluorophotometer has been set to read at 25 units with a solution of 0.00005 mg. of sodium fluorescein per ml. of water. With a Pfaltz and Bauer Fluorophotometer, use a No. 5113 in filter holder No. 6254, No. 3389 between the lamp and the cuvet housing, and No. 3486 in the cuvet holder.

Riboflavin and thiamine simultaneously. Riboflavin and thiamine can be determined in mixtures simultaneously by fluorescence.²⁷⁸ See thiamine, page 435.

Direct reading. Dissolve a sample containing 0.2-0.4 mg. of riboflavin in 20 ml. of a phosphate buffer for pH 4. Add 0.1 ml. of 5% aqueous sodium dithionite. Read at 445 m μ after 1 minute. Aerate for 1 minute and reread at 445 m μ .

AZORIBAMINE

A method for the estimation of azoribamine, an intermediate in the synthesis of riboflavin, is based on the extraction of the total coloring matter of the sample with acetone. The yellow coloring matter is extracted from another portion of the sample with benzene. The azoribamine content of the sample is measured as the difference between the acetone and benzene reading.²⁷⁹

Procedure—Extract one 20-mg. sample into 100 ml. of acetone and one 20-mg. sample into 100 ml. of cooled benzene. Filter, and read each at 470 m μ . Calculate the color content in each from a calibration curve. The acetone solution gives the total coloring matter in the sample and the benzene solution gives the yellow pigment only. To determine the azoribamine content, subtract the benzene value from that in acetone.

CARBAZOLE

In the presence of hydrochloric acid, carbazole reacts with xanthidrol and glacial acetic acid to form a colored product, which is possibly a xanthylum salt.²⁸⁰ Although the absorption maximum occurs at 567 m μ , readings are taken at 525 m μ (cf. Vol. IV, pp. 314-15). Anthracene, phenanthrene, acridine, and dibenzothiophene do not interfere, even when present in concentrations equal to that of carbazole. The interference of phenols including 2-naphthol can be eliminated by extracting the sample with aqueous sodium hydroxide. Acetone reduces the color intensity. The following compounds also form a color with the reagent: *N*-acetylcarba-

²⁷⁸ W. E. Ohnesorge and L. B. Rogers, *Anal. Chem.* **28**, 1017-21 (1956).

²⁷⁹ V. M. Iosikova, *Zhur. Anal. Khim.* **10**, 191 (1955).

²⁸⁰ Gerald Gilbert, R. M. Stickel and H. H. Morgan, Jr., *Anal. Chem.* **31**, 1981-3 (1959).

zole, *N*-cyanoethylcarbazole, indole, 2-methylcarbazole, and *N*-methylcarbazole. High blanks indicate incomplete cleaning of glassware. The color is stable overnight. Beer's law is followed and the relative precision of the method is 0.6%.

In the range of 0.004-0.1 mg., carbazole may be determined in concentrated sulfuric acid by *p*-nitrosophenol.²⁸¹ Beer's law is followed in this range at 655 m μ . Polycyclic compounds, such as pyrene, anthracene, and benzo[*a*]pyrene, form colored solutions in concentrated sulfuric acid. There is no interference from phenol, anisole, *N,N'*-dimethylaniline, pyrrole, indole, naphthalene, 1-naphthol, 1-methoxynaphthalene, 1-naphthylamine, and azulene.

A brilliant blue color is formed when carbazole is treated with 3-methyl-2-benzothiazolone hydrazone.²⁸² Beer's law is followed at 586 m μ for 0.004-0.09 mg. of carbazole. There is no interference from anthanthrene, coronene, benzoperylene, hydrocarbons, or benzaldehyde. Aliphatic aldehydes, aromatic amines, and imino heteroaromatic compounds react. In 80% sulfuric acid solution, carbazole is read at 430 m μ .²⁸³ For carbazoles along with *N,N*-dialkylanilines and diphenylamines by 5-nitroisatin, see page 409.

Procedure—*By xanthydrol in glacial acetic acid. Coal tar fractions.* To prepare the reagent, dissolve 5 grams of xanthydrol in glacial acetic acid and dilute to 1 liter with glacial acetic acid. To prepare the hydrochloric acid reagent, dilute 50 ml. of concentrated hydrochloric acid to 1 liter with glacial acetic acid.

Dissolve a sample containing 50 mg. of carbazole in glacial acetic acid, warming if necessary, and dilute to 100 ml. with glacial acetic acid. To a 1-ml. aliquot, add 5 ml. of xanthydrol reagent and 5 ml. of hydrochloric acid reagent. Wash down the sides of the flask with 10 ml. of glacial acetic acid. Heat at 100° for 15 minutes. Cool rapidly to room temperature and dilute to 100 ml. with glacial acetic acid. Read at 525 m μ .

By p-nitrosophenol. Dissolve a sample containing 0.01-0.3 mg. of carbazole by shaking with 1 ml. of concentrated sulfuric acid containing 0.5% of *p*-nitrosophenol. After 5 minutes, dilute to 10 ml. with concentrated sulfuric acid and read at 655 m μ within 15 minutes.

²⁸¹ Eugene Sawicki, Thomas R. Hauser, Thomas W. Stanley, Walter Elbert and Frank T. Fox, *ibid.* **33**, 1575-9 (1961).

²⁸² *Loc. cit.*

²⁸³ L. Sommer, *Chem. Listy* **47**, 1415-6 (1953).

By 3-methyl-2-benzothiazolone hydrazone hydrochloride. To 1 ml. of methanol solution of carbazole, add 1 ml. of 0.4% 3-methyl-2-benzothiazolone hydrazone hydrochloride solution and 2 ml. of 1% ferric chloride solution. Shake, and let stand for 30 minutes. Dilute to 10 ml. with a 1:5 mixture of concentrated hydrochloric acid and methanol. Read at 586 m μ .

In sulfuric acid. To 3 ml. of a methanol solution of carbazole, add 4-5 ml. of concentrated sulfuric acid. After 15 minutes, dilute with 80% sulfuric acid, and after 15 minutes, read at 430 m μ .

METHYPRYLON, 3,3-DIETHYL-2,4-DIOXO-5-PIPERIDINE

Methylprylon is determined as molybdenum blue.²⁸⁴

Procedure—Dilute Folin-Ciocaltu reagent (Vol. III, p. 116) with 2.5 volumes of water. Mix 1 ml. of sample with 1 ml. of reagent and 1 ml. of 4% sodium hydroxide solution. After 20 minutes, read at 600 m μ .

PYRIDONE, N-METHYL-2-PYRIDONE-5-CARBOXAMIDE

Passage of a urine sample through both an anion- and cation-exchange column remove most of the ultraviolet-light-absorbing substances except pyridine. The N-methyl-2-pyridone-5-carboxamide is eluted with water and read in the ultraviolet.²⁸⁵ N-Methyl-2-pyridone-3-carboxamide interferes. Nicotinamide, nicotinic acid, trigonelline, nicotinic acid, quinolinic acid, N-methyl-2-pyridone-5-carboxylic acid, N-methyl-2-pyridone-3-carboxylic acid, and N-methyl-2-pyridone do not interfere.

Pyridone may be determined in the same sample as N-methyl-nicotinamide, both metabolites of nicotinic acid in urine. N-Methyl-nicotinamide is separated from N-methyl-2-pyridone-5-carboxamide by passage through activated Decalso at pH 4.5-7. Since N-methyl-2-pyridone-3-carboxamide is not present in urine samples, it does not interfere. However, it forms a blue color with the reagent when present in other types of samples.

Another method is based on the conversion of the carboxamide group into an amino group by hypobromite and the diazotization and coupling

²⁸⁴ Satoru Yomogita and Takashi Arima, *Kagaku Keisatsu Kenkyusho Hokoku* 13, 36-7 (1960).

²⁸⁵ J. M. Price, *J. Biol. Chem.* 211, 117-24 (1954).

of the amino compound to form a purple azo dye.²⁸⁶ Phenol removes excess bromine. Interfering substances in urine are removed by shaking the sample with lead hydroxide. A more complete purification method involves adsorption of the *N*-methyl-2-pyridone-5-carboxamide on Lloyds reagent, eluting with sodium hydroxide and shaking the eluate with lead hydroxide. Errors due to incomplete recovery are avoided by the use of an internal standard. The procedure determines 0.001-0.05 mg. Up to 4 mg. of urea does not interfere.

Procedure—*In the ultraviolet. Urine.* Allow Dowex 1, 10% cross-linkage, 200-400 mesh, chloride form, to sediment in water. Decant the very fine and very coarse particles. Convert the resin to the hydroxide form by washing 750 ml. of packed resin with 24 liters of 8% sodium hydroxide solution at a flow rate of 500 ml. per hour. Wash with 5 liters of water. Sediment Dowex 50, 12% cross-linkage, 200-400 mesh, in the same manner. Wash 1500 ml. of packed resin with 25 liters of 2:1 hydrochloric acid, 12 liters of 1:2 hydrochloric acid, and 10 liters of water at a flow rate of 750 ml. per hour.

To prepare the chromatographic columns, seal a glass tube, 1.2×30 cm., to the bottom of a 125-ml. Erlenmeyer flask. Operate the columns in groups of 8 with air pressure distributed with a manifold. Add the resins to the columns to form superimposed layers consisting of, from the bottom up, 6 cm. of Dowex 1, 4 cm. of Dowex 50, 8 cm. of Dowex 1, and 5 cm. of Dowex 50. Pack each layer down before addition of the next layer by forcing water through the column with air pressure. Leave 5 cm. of water in the column when adding resin, to prevent mixing of layers. Wash the completed column with 100 ml. of water.

Collect the 24-hour sample in a bottle containing 25 ml. of toluene and store at 0° until used. Take two aliquots consisting of 1% of the total sample. Add 1 ml. of water containing 0.245 mg. of synthetic pyridone to one aliquot and dilute both aliquots to 30 ml. with water.

Add the samples to the columns and wash through with 10-20 ml. portions of water to a final volume of 100 ml. Force the sample and washings through the columns at a rate of 30-40 drops per minute. Read the combined effluent against a water blank at 258, 295, and 310 m μ .

In samples containing protein, add 2 grams of solid trichloroacetic acid per 100 ml. of urine. Filter after 10 minutes and proceed as above, using only an aliquot equivalent to 0.7% of the sample in place of 1%.

²⁸⁶ W. I. M. Holman, *Biochem. J.* **56**, 513-20 (1954).

By diazotizing and coupling with *N*-1-naphthylethylenediamine dihydrochloride. Collect 24-hour specimens under toluene and store at room temperature. Add a suitable sample to each of two centrifuge tubes. To tube 2, add a known amount of *N*-methyl-2-pyridone-5-carboxamide, usually 0.05 mg. To each tube, add 2 ml. of 33% lead nitrate solution and sufficient 10% sodium hydroxide solution to form lead hydroxide and to raise the pH of the solution to 9.2. Test the pH with external indicators or indicator papers, with 3.8% borax solution as a standard. Add the sodium hydroxide solution, usually 1.3 ml., slowly with frequent mixing and shake for intervals for 15 minutes, checking the pH at each interval. Dilute to 10 ml. with water. Mix, centrifuge, and filter. Transfer 1 ml. from tube 1 to each of two test tubes—test and test blank—and 1 ml. of tube 2 to each of two test tubes—recovery and recovery blank. Dilute each to 3 ml. with water.

To prepare the bromine solution, dissolve 12.5 grams of sodium bromide and 12.5 grams of bromine in water, and dilute to 100 ml. The reagent is stable in the refrigerator for 2-5 months. To prepare the sodium hypobromite reagent for pyridone, dilute 4 ml. of 10% sodium hydroxide solution to 45 ml. with water, add 2 ml. of bromine solution, and dilute to 50 ml. with water. This reagent is not stable for more than 24 hours in the dark.

Dilute the sample and the recovery tube containing up to 0.05 mg. of *N*-methyl-2-pyridone-5-carboxamide at pH 7-9 to 4 ml. with water. In the dark, add 1 ml. of sodium hypobromite solution, and after 4 minutes, 1 ml. of 0.45% phenol solution to remove excess bromine. Stopper, and hold the stopper in place with a metal cap. Heat for 10 minutes at 100°. Cool in running water for 4 minutes and remove the metal cap. Add 0.5 ml. of 1:4 hydrochloric acid and 0.5 ml. of 0.1% sodium nitrite solution. After 4 minutes, add 0.5 ml. of 0.5% ammonium sulfamate solution. After 4 minutes, add 0.5 ml. of 0.1% *N*-1-naphthylethylenediamine dihydrochloride solution. Stopper and shake well immediately after each addition. Read at 590 m μ after at least 1 hour.

In the case of the two blank solutions, dilute the hypobromite 1:1 with water and add the phenol 4 minutes before the hypobromite to eliminate the red color formed by urine samples. Calculate as follows:

N-methyl-2-pyridone-5-carboxamide in mg. per 24 hours

$$= \frac{(T - B_t)}{(R - B_r) - (T - B_t)} \times 1.44 \times \frac{A}{M}$$

in which T and R are extinctions of the test and recovery tubes; B_t and B_r are extinctions of the test blank and recovery blank; A is the amount in micrograms of the test substance added to the recovery tube; and M is the number of minutes excretion to which the sample of urine taken is equivalent.

Alternatively, measure two suitable samples into centrifuge tubes. To tube 2, add a known amount of *N*-methyl-2-pyridone-5-carboxamide, usually 0.025 mg. To each tube, add 0.5 ml. of 3% acetic acid and dilute each to 10 ml. with water. Add 0.5 ml. of 1:5 hydrochloric acid and 0.2 gram of Lloyds reagent, a highly adsorptive aluminum silicate. Stopper and shake for 10 minutes. Centrifuge, and decant the supernatant liquid. Wash the Lloyds reagent in the tubes with 5 ml. of 1:110 hydrochloric acid by mixing the contents and decanting. Drain for 10 minutes. Wipe off any fluid remaining in the neck of the tubes and add 4 ml. of 0.8% sodium hydroxide solution. Stopper, shake for 10 minutes, and centrifuge. Check to determine if the total volume is the same in both tubes. Transfer 3 ml. of the supernatant liquid in each tube to two new tubes containing 2 ml. of 33% lead nitrate solution. Adjust the pH to 9.2 with 10% sodium hydroxide solution, approximately 1.2 ml., and dilute to 10 ml. with water. Mix, centrifuge, and filter. Take two 3-ml. portions of the filtrate from tube 1 for analysis of the test and test blank solutions, and two 3-ml. portions from tube 2 for analysis of the recovery and recovery blank solutions. Develop as for the prior technic, starting at "Dilute the sample and the recovery tube . . ."

AZACYCLONOL, α -(4-PIPERIDYL)BENZHYDROL

Azacyclonol is developed with sodium β -naphthoquinone-4-sulfonate in the presence of sodium carbonate.²⁸⁷

Procedure—Urine. Add a 10-ml. sample to 4 grams of ammonium sulfate and 8 ml. of 10% sodium hydroxide solution. Mix, and extract with chloroform. Wash the chloroform layer with the Clark-Lubs buffer at pH 9 (Vol. I, p. 175). Extract with 5 ml. of 1:110 hydrochloric acid. To the hydrochloric acid extract containing the test substance, add 1 ml. of 0.3% sodium β -naphthoquinone-4-sulfonate solution and 1 ml. of 10% sodium carbonate solution. Let stand for 1 minute at room temperature.

²⁸⁷ Kiihno Kakemi, Toyozo Uno and Hajime Yamashina, *Yakugaku Zasshi* 79, 440-3 (1959).

Cool to 0° and extract with carbon tetrachloride. Dry the carbon tetrachloride layer with anhydrous sodium sulfate and read at 428 m μ .

Aqueous samples. Follow the procedure for urine samples, using a 5-ml. aqueous sample. Substitute chloroform for carbon tetrachloride to extract the color. Read at 458 m μ .

KYNURENIC ACID, 4-HYDROXYQUINALDIC ACID, AND
XANTHURENIC ACID, 4,8-DIHYDROXYQUINALDIC ACID

Kynurenic acid, quinaldic acid, 4-quinolone and *N*-methyl-4-quinolone, urinary metabolites of tryptophan are read in the ultraviolet after separation and purification on ion-exchange resins.²⁸⁸ For separation of kynurenic acid from related tryptophan metabolites and reading by fluorescence, see kynurenine, page 294.

Kynurenic acid, xanthurenic acid, and the 8-methyl ester of the latter are separable from urine in a highly purified form on a column of Dowex 50 in the hydrogen form.²⁸⁹ Xanthurenic acid is read by its fluorescence in strong alkali; kynurenic acid is read similarly in strong sulfuric acid. Neither interferes with the other. Xanthurenic acid is a normal metabolite of tryptophan. Quinaldic acid is not eluted from the column in the procedure. The 8-methyl esters of xanthurenic acid and 8-hydroxyquinaldic acid are eluted but do not interfere. Xanthurenic acid is also coupled with *p*-diazobenzenesulfonic acid in a borate buffer for pH 7 and read at 496 m μ .²⁹⁰

Procedure—*In the ultraviolet. Urine.* Prepare Dowex 50 as described under "*N*-Methyl-2-pyridone-5-carboxamide" on page 552. Sediment a slurry of Dowex 1, chloride form, 10% cross-linked 200-400 mesh to remove very coarse and very fine particles.

Take a sample equivalent to 5% of the 24-hour human urine sample, 1% of dog urine, 10% of cat or rat urine samples, and add to two flasks. Adjust the pH of the sample to 8.5-9 with a few drops of a saturated sodium hydroxide solution. Remove the flocculent precipitate by filtration. To one, add 0.610 mg. of kynurenic acid, 0.558 mg. of quinaldic acid, and 0.468 mg. of 4-quinolone. Adjust the volume with water to 150 ml. for

²⁸⁸ R. R. Brown and J. M. Price, *J. Biol. Chem.* **219**, 958-97 (1956).

²⁸⁹ J. M. Price and L. W. Dodge, *J. Biol. Chem.* **223**, 699-704 (1956); Kiyoo Sotoh and J. M. Price, *ibid.* **230**, 781-9 (1958).

²⁹⁰ M. Kaleb, *Acta Univ. Palackianae Olomuc.* **23**, 125-32 (1961).

the human sample, to 100 ml. for the cat sample, to 60 ml. for the dog sample, or to 50 ml. for the rat sample.

Pass the sample through a 3.3-cm. column of Dowex 1 at the rate of 30-40 drops per minute. Wash with 150 ml. of water. Reserve the effluent of the sample and washings for analysis for 4-quinolone and *N*-methyl-4-quinolone. Wash the column with 150 ml. of 0.05 *M* monochloroacetic acid buffer at pH 3 to remove quinaldic acid and with 425 ml. of 0.1 *M* monochloroacetic acid buffer in 25% ethanol at pH 3 to elute kynurenic acid.

Acidify the 4-quinolone fraction to 0.3 *N* with hydrochloric acid. Pass through a 1.9-cm. column of Dowex 50. Wash with 50 ml. of water and 50 ml. of 1:3 hydrochloric acid. Elute with 200 ml. of 1:1 hydrochloric acid. Operate the columns without pressure. The 4-quinolone and *N*-methyl-quinolone are eluted together.

Acidify the quinaldic acid fraction to 0.3 *N* with hydrochloric acid and pass through a 1.9-cm. column of Dowex 50. Wash with 50 ml. of water and 50 ml. of 1:10 hydrochloric acid. Elute with 150 ml. of 1:3 hydrochloric acid. Operate the columns without pressure.

Adjust the pH of the kynurenic acid fraction to 8.5-9 with a saturated sodium hydroxide solution and pass through a 3.3-cm. column of Dowex 1. Wash with 50 ml. of water and 50 ml. of 1:240 hydrochloric acid. Elute the kynurenic acid with 250 ml. of 1:3 hydrochloric acid. Read the quinaldic acid at 247 and 322 $m\mu$ and the kynurenic acid at 245 and 311 $m\mu$. Read the 4-quinolone sample at 229 and 300 $m\mu$. Xanthurenic acid was left on the Dowex 1.

By fluorescence. Urine. The ion-exchange column is 1.2×15 cm. and contains 3 cm. of Dowex 50 in the hydrogen form. Dilute an appropriate sample of urine to 120 ml. with water. Add to the column and wash with 50 ml. of 1:50 hydrochloric acid, 100 ml. of 1:20 hydrochloric acid, and 20 ml. of water. Then elute with 396 ml. of water. Add 4 ml. of 0.5 *M* phosphate buffer for pH 7.4 and adjust the volume to 400 ml.

Take 1-6 ml. of the treated sample and adjust to 6 ml. with 0.005 *M* phosphate buffer for pH 7.4. Slowly add 4 ml. of concentrated sulfuric acid. Mix and cool. Activate at 340 $m\mu$ and read the fluorescence of kynurenic acid at 435 $m\mu$ after 1 hour. The fluorescence is stable for 2 hours.

For xanthurenic acid, adjust 1-5 ml. of the treated sample to 5 ml. with 0.005 *M* phosphate buffer for pH 7.4. Add 5 ml. of saturated solution of sodium hydroxide. Centrifuge for 5 minutes. After 1 hour, activate

at 370 $m\mu$ and read at 530 $m\mu$. The fluorescence is stable for at least 2 hours.

PENTYLENETETRAZOL

Pentylenetetrazol is precipitated from aqueous solution by cuprous chloride reagent. The filtered pentylenetetrazol-cuprous chloride complex is dissolved in concentrated nitric acid. The acid decomposes the complex and simultaneously oxidizes the cuprous salt to the cupric form. After adjustment of the solution to a pH of 4, the deep blue color of the copper is developed with tetraethylenepentamine.²⁹² Ingredients usually present with pentylenetetrazol do not interfere. Ephedrin is not precipitated. Quinine sulfate and quinidine sulfate form yellow flocculent precipitates with the cuprous chloride reagent, and therefore, these alkaloids do interfere and must be separated by chromatography. Beer's law is followed for 2-10 mg.

Sample—Tablets. Determine the average weight of 20 tablets. Pulverize and dissolve a sample containing approximately 100 mg. of pentylenetetrazol in water and dilute to 100 ml. Stopper, mix, and filter through paper, discarding the first portion of the filtrate. Develop 5 ml. of the filtrate containing approximately 5 mg. of pentylenetetrazol with cuprous chloride.

Liquids. Dilute an aliquot of the sample containing approximately 100 mg. of pentylenetetrazol to 100 ml. with water. Stopper, and mix well. Develop a 5-ml. aliquot with cuprous chloride.

Procedure—Dissolve 1.25 gram of cuprous chloride, 10 grams of ammonium chloride, and 1 gram of sodium meta-bisulfite in water, and dilute to 100 ml. To prepare the acetate buffer, dissolve 16.6 grams of anhydrous sodium acetate in 100 ml. of water, add 24 ml. of glacial acetic acid, and dilute to 200 ml.

Dilute the sample containing 2-10 mg. of pentylenetetrazol to 10 ml. with water. Add 2 ml. of the cuprous chloride reagent. Stopper, and place in an ice bath for 20 minutes with swirling at 5-minute intervals. Filter the precipitated pentylenetetrazol-cuprous chloride complex with suction through a sintered glass filter funnel of medium porosity, and discard

²⁹² Robert A. Daoust, *J. Pharm. Sci.* **52**, 642-4 (1963).

the filtrate. Wash the precipitate twice with 5-ml. portions of cold 1% acetic acid solution and discard the washings.

Dissolve the residue directly from the sintered glass funnel in 1 ml. of concentrated nitric acid. Collect the filtrate in a test tube placed inside a flask attached to the funnel. Wash the walls and plate of the funnel with 5 ml. of cold water and add to the filtrate.

Remove the filtrate, repeat the washing with another 5 ml. of cold water, and add the washing to the filtrate. Add 10 ml. each of acetate buffer, 6% sodium hydroxide solution, and 2% tetraethylenepentamine solution in succession to the sample. Dilute to 50 ml. with water. Stopper, and mix well. Let stand for 10 minutes and read the copper tetraethylenepentamine complex at 580 $m\mu$.

DIPYRIDAMOLE, 2,2',2'',2'''-[(4,8-DIPIPERIDINEOPYRIMIDO
[5,4, α] PYRIMIDINE-2,6-DIYL) DINITRILO]TETRAETHANOL

Dipyridamole and its metabolites are determined in body fluids and tissues spectrofluorimetrically.²⁹³ Beer's law applies at 0.002-2 microgram per ml. Both mono- and diglucuronides are hydrolyzed by the technic given.

Procedure—*Plasma, bile, or urine. Free dipyridamole.* Mix 0.1-10 ml. of sample with 1 ml. of 12.114% 2-amino-2-hydroxymethyl-1,3-propanediol buffer at pH 8.6. Add 40 ml. of water-saturated ether and shake for 20 minutes. Centrifuge. Shake 30 ml. of the ether layer for 10 minutes with 5 ml. of 1:110 hydrochloric acid. Adjust 4 ml. of the acid layer to pH 9. After 10 minutes, excite at 305 $m\mu$ and read at 505 $m\mu$.

Total dipyridamole. Incubate the sample with equal volumes of 6.8 sodium acetate trihydrate buffer for pH 4.7 and β -glucuronidase (Keto-dase at 5000 units per ml.) overnight at 37°. Adjust the pH to 8.6 and proceed with a 0.1-10-ml. sample as for free dipyridamole, from "Add 40 ml. of water-saturated ether . . ."

Tissue. Homogenize 1 gram with 9 ml. of 1:550 hydrochloric acid. Treat 5 ml. of the homogenate as described for plasma.

²⁹³S. B. Zak, H. H. Tallan, G. P. Quinn, I. Fratta, and P. Greengard, *J. Pharmacol.* **141**, 392-8 (1963); Sylvia B. Zak, H. H. Tallan, Gertrude P. Quinn, I. Fratta, and P. Greengard, *J. Pharmacol. Exptl. Therap.* **141**, 392-8 (1963).

TMB-4, *N,N'*-TRIMETHYLENE*bis*(4-FORMYLPYRIDINIUM
BROMIDE) DIOXIME

This compound is developed by chloramine-T in cyanide solution followed by addition of barbituric acid.²⁹⁴

Procedure—Mix 1 ml. of 1% potassium cyanide solution, 5 ml. of 1% chloramine-T solution, and 2 ml. of 1:100 hydrochloric acid. To this, add 0.5-3 ml. of sample containing around 0.2 mg. of test substance per ml. Dilute to 13 ml. with water. After 10 minutes, add 10 ml. of 1% barbituric acid solution. Set aside for 10 minutes, then heat at 75° for 10 minutes. After 10 minutes to cool, dilute to 25 ml. and read at 642 m μ .

BETAINES

Ethylene dichloride solutions of periodides of *betaine*, *trigonelline*, *carnitine*, *stachydrine* and γ -*butyrobetaine* absorb in the ultraviolet with maxima at 365 and 295 m μ .²⁹⁵ Better periodide yields are obtained in sulfuric acid than in hydrochloric acid. A longer reaction period combined with use of hydrochloric acid gives satisfactory results. Histidine dichloride does not interfere. When the concentration of iodine is 17.5 grams per 100 ml., a periodide precipitate occurs with 0.08 mg. of nicotinic acid. Adenine interferes. When the sample is an effluent of chromatographic assay, these compounds do not interfere. In the presence of hexamine, betaine can be precipitated as the reineckate.²⁹⁶

Procedure—To prepare the reagent, dissolve 15.7 grams of iodine and 20 grams of potassium iodide in water and dilute to 100 ml. A 0.5-ml. sample should contain 0.01-0.1 mg. of the quaternary nitrogen compound in 1:35 sulfuric acid or 1:10-1:1 hydrochloric acid. The acid content may vary. However, standards of the same acid concentration should be run with the sample. Cool the sample to 0-4° for 10 minutes. Add 0.2 ml. of cold iodine-potassium iodide reagent. Mix, and keep at 0-4° for 80 minutes. To hasten crystallization of the periodides, scratch the sides of the tube frequently and stir with a tapered rod. Centrifuge for 5 minutes at 4000 rpm. in a refrigerated centrifuge. Aspirate the supernatant liquid.

²⁹⁴ J. Vacek, *Cesk. farm.* **11**, 464-6 (1962).

²⁹⁵ Joseph S. Wall, Donald D. Christianson, Robert J. Dimler and Frederick R. Senti, *Anal. Chem.* **32**, 870-4 (1960).

²⁹⁶ M. Mosse, *Pharm. Acta Helv* **33**, 80-4 (1958).

Avoid disturbing the precipitate. For samples containing 0.01-0.05 mg. of betaines, dissolve in 5 ml. of ethylene dichloride. For samples containing 0.02-0.1 mg., dissolve in 10 ml. Read at 365 $m\mu$.

2-MERCAPTOBENZOTHAZOLE

The reaction product of 2-mercaptobenzothiazole and sodium hydroxide is dissolved in sodium borate and read at 309 $m\mu$.²⁹⁸ The method is designed for assay of the impure commercial product.

Procedure—Dissolve a sample containing about 0.25 gram of 2-mercaptobenzothiazole in 25 ml. of 2% sodium hydroxide solution by heating at 50-60° for 10-15 minutes. Cool, and dilute to 500 ml. Filter, and dilute a 15-ml. aliquot to 500 ml. with 0.38% sodium borate solution. Read at 309 $m\mu$ against the borate solution.

PURINES, ETC.

It is necessary to combine the determination of several compounds here because they are frequently associated and require separation for determination by the same method. Thus, *adenine* (cf. Vol. IV, pp. 336-7), *cytosine* (cf. Vol. IV, p. 296), *thymine* (cf. Vol. IV, pp. 295-6), and *guanine* (cf. Vol. IV, pp. 337-40) are precipitated from a solution of purines and pyrimidines by silver perchlorate. The solution is read at 262 $m\mu$ and 280 $m\mu$ before and after this precipitation, to give a measure of their content.²⁹⁹ The success of the method depends on the quantitative separation of the mixture into purines and pyrimidines.

Adenine, guanine, cytosine, *uracil*, *hypoxanthine* and *inosine* are read in the ultraviolet after separation from the sample by passage through resin in the formate and chloride forms.³⁰⁰ Nucleotides are removed on the resin on the formate form. The purines and pyrimidines are removed from the resin in the chloride form at pH 11-12. They are eluted by a gradient of increasing chloride and tetraborate concentrations and diminishing pH.

Uranyl nitrate complexes the adenine in blood samples. The uranium

²⁹⁸ Ya. A. Gurvich and V. P. Kostikova, *U.S.S.R. Patent 144,636* (15.2.62).

²⁹⁹ C. F. Emanuel and I. L. Chaikoff, *Biochim. et Biophys. Acta* **28**, 550-61 (1958).

³⁰⁰ N. R. Jones, *Analyst* **85**, 111-115 (1960).

is precipitated with ammonium hydroxide and the adenine is developed with silver nitrate.³⁰¹ Guanine does not interfere.

A yellow color appears upon the oxidation of adenine by permanganate.³⁰² The following compounds do not interfere: guanine, hypoxanthine, xanthine, purine, uric acid, caffeine, theobromine, theophylline, 6-chloropurine, 6-mercaptapurine, uracil thymine, cytosine, 5-methylcytosine. Adenine-containing compounds such as adenosine, adenylic acid, adenosine di- and triphosphate all produce the yellow color with an absorption peak at 330 m μ . Beer's law is followed for 0.01-0.05 mg. of adenine per ml. of final solution.

To determine purines in nucleic acids, extract chloride ion and purines other than those in nucleic acids with 80% ethanol slightly acidified with sulfuric acid. After hydrolysis of the residue, the purines are separated by precipitation with silver nitrate, freed by treatment with hydrochloric acid, and separated by paper chromatography for reading adenine and guanine in the ultraviolet.³⁰³

After separation by paper chromatography, adenine is developed with an eosin solution saturated with mercuric chloride.³⁰⁴ Adenine, guanine, adenosine, guanosine, and adenylic and guanilic acids are also determined by their color reaction with 0.5% solution of bromophenol blue in 95% ethanol containing 10 ml. of 1:30 nitric acid per 100 ml.³⁰⁵ Adenine and guanine can be read nephelometrically.³⁰⁶ Adenine, guanine, and hypoxanthine are read in the ultraviolet by differential methods.³⁰⁷

6-Hydroxypurine has a maximum absorption at 250 m μ .³⁰⁸ 2,6-Dihydroxypurine has a maximum at 275 m μ and a minimum at 240 m μ . The value of each can be calculated.

Procedure—*In the ultraviolet. Cod muscle.* As the Dowex 1, formate column, attach a 100-ml. separatory funnel to a 4-cm. \times 1.8-cm. glass column by a rubber or ground glass seal. Add a glass wool plug to the bottom of the column. To prepare the Dowex 1-8X, formate, suspend

³⁰¹ Raoul Lecoq, *Ann. biol. clin.* **12**, 510-17 (1954); *Ann. pharm. franc.* **12**, 104-9 (1954).

³⁰² Joseph R. Davis and Robert N. Morris, *Anal. Biochem.* **5**, 64-9 (1963).

³⁰³ I. W. McDonald, *Biochem. J.* **57**, 566-8 (1954).

³⁰⁴ J. P. Varma, *J. Sci. Ind. Research* **20B**, 511-2 (1961).

³⁰⁵ Lavin Popa, *Acad. Rep. Populare Romane, Inst. Biochim., Studii Cercetari Biochim.* **4**, 123-8 (1961).

³⁰⁶ Wanda Leyko and Bronislav Filipowicz, *Roczniki Chem.* **29**, 1095-1101 (1955).

³⁰⁷ A. I. Komkova and V. A. Rogozkin, *Vop. Med. Khim.* **7**, 540-2 (1961).

³⁰⁸ S. Triccerri and A. Braschi, *Farmaco (Pavia) Ed. sci.* **12**, 28-33 (1957).

Dowex 1-8X, chloride, 200-400 mesh, in water and pour into the detached column until a bed 2 cm. deep is obtained. Wash the bed with ten times its volume of a solution containing 28% of formic acid and 6.8% of sodium formate, next with a similar volume of 88% formic acid, and then with water until the effluent is free from acid. Do not allow the column to run dry.

To prepare the Dowex 1, chloride apparatus, connect a 500-ml. separatory funnel to a 250-ml. conical flask fitted near the base with a right-angled side tube 3 mm. in diameter and 15 cm. long. The 250-ml. flask contains a glass-sheathed stirrer bar and is placed on a small magnetic stirrer motor. Add 5 cm. of polythene tubing between the side-arm of the 250-ml. flask and a 4-cm. length of glass tubing that leads through a rubber stopper into a glass column, 15 × 1.3 cm. Fit the column with a two-way tap at the base and a glass wool plug inserted immediately above the tap. Fit a small right-angled side-arm to the column 3 cm. from the top. Attach a short length of rubber tubing and fit both this tubing and the polythene tubing with screw clamps.

To prepare the Dowex 1-8X, chloride, suspend the resin in water and pour the slurry into the detached column to form an 8-cm. bed. Wash with 50 times its volume of 2:5 ammonium hydroxide solution and with water until the effluent is free from ammonia. Wash with 20 times its volume of 1:5 hydrochloric acid and again with water until free from acid. Repeat the ammonia and acid washing. Chill a Büchner funnel in ice. Wash the filter paper before use in 1:10 hydrochloric acid and with water to remove material absorbing at 260 m μ .

Homogenize a 6-10 gram sample with 2 volumes of chilled 6% perchloric acid. Filter rapidly at 0° and immediately adjust the pH to 6.5 with 28% potassium hydroxide solution. Do not allow the pH to rise above this value, as the nucleotides may break down. After 30 minutes at 0°, remove any potassium perchlorate that has crystallized.

To remove the nucleotides, run an aliquot equivalent to 5 grams of tissue through the column of Dowex 1, formate. Wash with water until the optical density of the effluent has decreased to 0.01 at 260 m μ . The effluent and washings contain the nucleoside, purine, and pyrimidine fractions. Adjust the pH of the effluent and washings to 11-12 with 1:9 ammonium hydroxide. Run through the column of Dowex 1, chloride, from the separating funnel. Wash with water containing 2 drops of 1:9 ammonium hydroxide solution per 50 ml. until the optical density of the effluent has decreased to 0.01 at 260 m μ . Attach the column to the assembly and with the polythene connection closed, pour 200 ml. of a solution

of 1:9 ammonium hydroxide containing 0.4 ml. of 1:1 hydrochloric acid into the 250-ml. flask. Attach the separating funnel to the assembly and fill it with 1:240 hydrochloric acid containing 1% of sodium tetraborate. Start the stirrer. Open the separating funnel stopcock and the polythene connection, and by means of a screw clamp on the side-arm of the column, release sufficient air to break the air-lock between the column and the 250-ml. flask. Open the column stop-cock and start the fraction collector to collect 5-ml. fractions. Collect 90 5-ml. fractions over 2-16 hours. Read at 260 $m\mu$ to establish the positions of the peaks, and combine the fractions of individual peaks. Add hydrochloric acid to make the solution 1:5, and make the optical density measurements against the known molar extinction coefficients.

<i>Base</i>	<i>Absorption maximum, $m\mu$</i>	<i>Molar extinction coefficient ($\times 10^{-3}$)</i>
Cytosine	276	10.0
Uracil	260	8.2
Guanine	275	7.35
Adenine	262	13.15
Hypoxanthine	248	10.8
Inosine	251	10.9

Protozoa, bacteria, plant leaves, feedstuffs, digesta. Extract samples containing about 1 mg. of purine nitrogen with 80% ethanol made 0.001 *N* with sulfuric acid. Treat moist samples with absolute ethanol to a final concentration of 80% of ethanol. Filter with suction over a thin layer of Celite and wash twice with 80% ethanol, once with absolute ethanol, and once with acetone. Remove the acetone with suction and dry in an oven at a low temperature.

Hydrolyze the sample in 17 ml. of 1:35 sulfuric acid by boiling for 1 hour at 100°. Separate from the fibrous residue by straining through stainless steel gauze in a syringe. Wash with water. Dilute the filtrate and washings to 25 ml. and filter. Add 1 ml. of 8.5% silver nitrate solution to 20 ml. of the filtrate and let stand for 1-2 hours in a refrigerator. Wash down the adherent precipitate with 1 ml. of a saturated silver sulfate solution in 1:3500 sulfuric acid. Centrifuge at 0° and remove the supernatant liquid. Chill, and repeat the centrifuging at 0°. Take up the precipitate in small amounts of 1:10 hydrochloric acid and dilute to 1 ml. with 1:10 hydrochloric acid. Centrifuge to remove the silver chloride. Separate the purines in this solution on Whatman No. 3 paper, using a 77:13:10 mixture of butanol-water-formic acid in the descending direc-

tion, and identify the spots under ultraviolet light. Cut out the spots, extract with 1:110 hydrochloric acid, and read at 250 $m\mu$ for guanine and 262 $m\mu$ for adenine.

Blood. To prepare the buffer, dissolve 3 grams of sodium veronal, 2 grams of sodium acetate, and 0.1 gram of sodium benzoate in water, and dilute to 100 ml. As the silver reagent, dissolve 2 grams of silver nitrate in water and add concentrated ammonium hydroxide until the precipitate redissolves. Just before use, mix 1 part of this solution with 2 parts of 2% gum arabic solution and 1 part of the buffer.

Shake a 4-ml. oxalated or heparinized sample with 14 ml. of 10% trichloroacetic acid solution. Filter after 20 minutes and make 2 ml. of the filtrate alkaline with 4% sodium hydroxide solution, using phenolphthalein as an indicator. Acidify with 1 drop of 3% acetic acid solution and add 1 drop of 6.7% dibasic sodium phosphate solution. Add 0.5 ml. of 20% uranyl nitrate solution. Centrifuge for 15 minutes. Decant, and suspend the precipitate of an adenine-uranium complex in 5 ml. of water containing 1 drop of 6.7% dibasic sodium phosphate solution and 0.3 ml. of 20% uranyl nitrate solution. Centrifuge and decant. Dissolve the washed precipitate in 1 ml. of 6% sulfuric acid. Add 1 ml. of water and heat for 45 minutes at 100°. Cool, and add dropwise 10% ammonium hydroxide solution until the solution is alkaline to litmus. This precipitates the uranium. Dilute with water to 5 ml. and centrifuge. Mix 4 ml. of the supernatant liquid with 0.5 ml. of buffer and centrifuge. Mix 4 ml. of the supernatant liquid with 2 ml. of freshly prepared silver reagent. Dilute to 100 ml. with water and read the adenine after 2 minutes.

Tobacco-virus-infected leaf sap. Clear the sap by the chloroform-water technique. Precipitate the virus protein by adding 32% trichloroacetic acid solution. Hydrolyze 50 mg. of the washed and dried protein with 0.5 ml. of 1:10 hydrochloric acid at 100° for 1 hour. Chromatograph a 0.025-0.05-ml. portion on Whatman No. 1 filter paper with 1:14 hydrochloric acid containing 70% of *tert*-butanol. Develop with 0.2% eosin solution in 96% ethanol saturated with mercuric chloride. Elute the red-violet spots with 5 ml. of 1:110 hydrochloric acid and read at 248 $m\mu$ for guanine and 260 $m\mu$ for adenine.

Deoxyribonucleic acid. To prepare silver perchlorate, convert silver nitrate to silver hydroxide with a stoichiometric amount of sodium hydroxide. Wash the silver hydroxide thoroughly with water and mix with a

slight excess of 60% perchloric acid. Filter the colorless solution through a sintered disk and slowly concentrate over sulfuric acid in the dark until crystals form. Recrystallize the silver perchlorate 2-3 times from a small amount of hot water until a 20% solution has an absorbance at 250 $m\mu$ of not over 0.25.

To 10 ml. of deoxyribonucleic acid solution, add 20 ml. of 72% perchloric acid. After a few minutes, mix the charred mass well by rotation. Keep at room temperature for 45 minutes until the nucleic acid is completely dissolved. Stopper, and heat for 1 hour at 100°. Do not disturb during the heating period. Similarly heat a blank of perchloric acid. Cool in water and add 5 ml. of water to the sample and blank. Triturate the charred mass with a glass rod. Centrifuge, and remove 1 ml. of the supernatant liquid with a syringe. Dilute the 1 ml. to 50 ml. and read at 262 and 280 $m\mu$ against the blank to determine the total absorbancy. To two additional 1-ml. portions of the supernatant liquid, add 1 ml. of freshly prepared 10% silver perchlorate solution. Prepare a blank with 1 ml. of the heated perchloric acid blank and similarly add silver perchlorate. Mix with a glass rod. Add enough 3% sodium hydroxide solution, usually 0.55 ml., to bring the pH of the solutions to 2-2.7. Since the total volume of the mixture must be known, all volumes added should be measured. Pour each solution into centrifuge tubes and centrifuge immediately. The purines separate out as silver-purine precipitates. To two 2-ml. aliquots of the supernatant liquid containing the pyrimidines alone, add 1 drop of 70% perchloric acid and dilute to 25 ml. Read against the blank at 250, 262, and 280 $m\mu$ for the pyrimidine concentration. Subtract the pyrimidine values at 262 and 280 $m\mu$ from the total absorption values at 262 and 280 $m\mu$ to give the absorption derived from purines. Calculate as follows:

$$\begin{aligned}C_{\text{cytosine}} &= [1.38 A_s (280 m\mu) - 1.09 A_s (250 m\mu)] \times 10^{-4} \\C_{\text{thymine}} &= [2.59 A_s (250 m\mu) - 0.804 A_s (280 m\mu)] \times 10^{-4} \\C_{\text{guanine}} &= [2.45 A_s (280 m\mu) - 0.875 A_s (262 m\mu)] \times 10^{-4} \\C_{\text{adenine}} &= [1.30 A_s (262 m\mu) - 1.48 A_s (280 m\mu)] \times 10^{-4}\end{aligned}$$

in which C = concentration of the purine in moles per liter.

SUBSTITUTED PURINES AND ANTAGONISTS OF PURINES

Purines and purine antagonists are reduced by zinc amalgam in dilute acid to an amine that is diazotized and coupled with *N*-1-naphthylethylenediamine to form an intensely colored azo dye.³⁰⁹ Uric acid, hepa-

³⁰⁹ T. Li Loo and Marvin E. Michael, *J. Biol. Chem.* 232, 99-106 (1958).

rin, 5-hydroxyindoleacetic acid, insulin, trichloroacetic acid and pentobarbital do not interfere. Sulfonamides, aromatic amines, and compounds reducible to diazotizable amines interfere. A control blood sample should be carried through the procedure, since plasma gives a slight pink color in the method. Urine samples are treated differently to eliminate a source of error from a pink color formed with a maximum at 541 $m\mu$.

Beer's law is followed for 0.002-0.012 mg. of 6-mercaptapurine per ml. and for 4-aminopyrazole, 3,4-d-pyrimidine. Table 33 shows the absorption maximum and optical density at the maximum for purines and purine antagonists.

TABLE 33. AZO DYES DERIVED FROM PURINES AND PURINE ANTAGONISTS^a

<i>Compound</i>	λ_{max}	<i>Optical density at λ_{max}</i>	<i>Compound</i>	λ_{max}	<i>Optical density at λ_{max}</i>
	<i>mμ</i>			<i>mμ</i>	
Adenine	505	0.90	Pyrazolo(3,4- <i>d</i>)pyrimidines		
Adenosine	505	0.37	4-Amino	520	0.89
Caffeine		0.00	4-Mercapto	500	1.01
6-Chloropurine	505	0.43	4-Methylamino	500	0.37
Guanine		0.00	4-Hydroxy	505	0.38
Hypoxanthine	515	0.34	4-Hydroxy-6-amino		0.00
Isoguanine	505	0.40	6-Hydroxy-4-amino	495	0.31
2-Mercaptopurine	505	0.16	4,6-Dihydroxy		0.00
6-Mercaptopurine	505	0.84	1-Methyl-4-amino	485	0.49
6-Mercaptopurine D-riboside	505	0.20	1-Methyl-4-ethylamino	485	0.15
6-Methylmercaptapurine	505	0.26	1-Methyl-4-dimethylamino	485	0.23
6-Methylpurine	505	0.14	1-Methyl-4- <i>n</i> -propylamino	485	0.13
9-Methyl-6-chloropurine	495	0.29	1-Methyl-4- <i>n</i> -butylamino	485	0.13
Purine	502	1.02	1-Methyl-4(1',1',3',3'-tetramethylbutylamino)	485	0.19
Theobromine		0.00	Pyrazolo(4,3- <i>d</i>)pyrimidines		
Theophylline		0.00	7-Hydroxy	495	0.02
2,6,8-Trichloropurine	502	0.25	7-Mercapto	553	0.60
Xanthine	510	0.30	7-Hydroxy-3-methyl	545	0.15
Uric acid		0.00	7-Amino-3-methyl	548	0.54
			7-Hydrazino-3-methyl	545	0.28

^a Sample contains 0.01 mg. per ml. in 1:35 sulfuric acid processed according to the general procedure.

Sample—Blood. To prepare the 5% trichloroacetic acid solution, dissolve in 1 liter of 1:5 sulfuric acid. To 3 ml. of plasma prepared from heparinized blood, add 3 ml. of the trichloroacetic acid reagent and mix thoroughly. Centrifuge at 2000 rpm for 20 minutes. Develop a 3-ml. portion of the supernatant fluid.

Urine. Dilute a 1-ml. sample with 5 ml. of 1:5 sulfuric acid and make up to 10 ml. with water. Develop a 3-ml. aliquot, carrying out the reduction at 90° instead of at room temperature, with zinc amalgam. If 4-aminopyrazolo(3,4-d)pyrimidine is present, the urine must be extracted with an equal volume of ether before starting.

Procedure—To prepare the zinc amalgam, react 10 grams of granular zinc with 20 ml. of mercury at 150°. The amalgam is reusable until its density becomes higher than 13.5 grams per ml. Immediately after use, wash the amalgam thoroughly with water and then with acetone. Use red glassware to prevent the color from fading.

Shake a 3-ml. sample containing up to 0.01 mg. of the purine or purine antagonist per ml. in a shaking incubator at about 100 cycles per minute in a red flask with 1 ml. of zinc amalgam for 30 minutes. Use room temperature for blood samples, 90° for urine samples. Mix a 2-ml. portion of the reduced solution with 0.5 ml. of 0.2% sodium nitrite solution in a red test tube. After 3 minutes, add 0.5 ml. of 1% ammonium sulfamate solution and let stand for 10 minutes with occasional shaking. Add 0.5 ml. of 0.2% *N*-1-naphthylethylenediamine dihydrochloride solution and let stand for 10 minutes for color development. Read at the appropriate wave length, according to Table 33, against a 1:11 sulfuric acid blank that has been carried through the procedure.

VITAMIN B₄, ADENINE, 6-AMINOPURINE

Adenine is read in the ultraviolet after oxidation.³¹⁰ This method also applies to adenine nucleotides and nucleosides without prior hydrolysis. For other methods, see purines.

Procedure—To a 15-ml. sample, add 0.2 ml. of 1:1 sulfuric acid. Mix, and add 0.1 ml. of 0.24% potassium bromide solution. Shake, and add 0.3 ml. of 3.2% potassium permanganate solution. After 5 minutes, decolorize the permanganate by dropwise addition of 6% hydrogen peroxide solution. Adjust to 3 ml. with water and read at 330 mμ within 15 minutes.

6-MERCAPTOPURINE

Sodium nitroprusside is a reagent for 6-mercaptopurine.³¹¹ Hypoxanthine and glucose do not interfere. The method is accurate to within

³¹⁰ Joseph R. Davis and Robert N. Morris, *Anal. Biochem.* **5**, 64-9 (1963).

³¹¹ J. Vacek, *Czechoslov. farm.* **9**, 126-9 (1960); *The Prophylactics in Animal Feeds*

$\pm 2\%$. For the determination of 6-mercaptopurine by reduction, and the diazotization and coupling with *N*-1-naphthylethylenediamine to form an azo dye, see "Substituted purines" on page 565.

Procedure—A suitable sample contains 20 mg. of 6-mercaptopurine in 100 ml. of 0.4% sodium hydroxide solution. Dilute an aliquot with 0.4% sodium hydroxide solution to 5 ml. and add 1 ml. of 2% sodium nitroprusside solution. After 10 minutes, add 4 ml. of a 4:1 mixture of acetic acid and methanol. Dilute with 1 ml. of water and read the green color after 5 minutes with a red filter.

GUANINE, IMINOXANTHINE, 2-AMINO-6-HYDROXYPURINE

A rapid method for guanine in nucleic acid hydrolyzates, requiring no separation from adenine or pyrimidine derivatives, involves reading of the fluorescence of an alkaline solution.³¹² (cf. Vol. IV, p. 339-41). The fluorescence is measured at 340 $m\mu$ instead of at the 350 $m\mu$ peak, to avoid interference from methylaminoguanines that may be present. With this modification, guanine may be measured in the presence of as much as 5% dimethylaminoguanine. Nucleotides and nucleosides fluoresce mainly in acid solution. Thymine and adenine do not interfere. Additional methods for guanine are described in the section on purines, page 560.

Sample—*Nucleic acid solutions*. To a solution containing 0.05 mg. of nucleic acid, approximately equivalent to 0.005 mg. of guanine, add hydrochloric acid to a final concentration of 1:10. The final volume should be 1 ml. Hydrolyze at 100° for 30 minutes and cool. Neutralize an aliquot containing 0.0005-0.001 mg. of guanine with 4% sodium hydroxide solution and dilute to 2 ml. with 1:20 ammonium hydroxide. Read the fluorescence with excitation at 275 $m\mu$ and fluorescence at 340 $m\mu$.

PYRROLE-2-CARBOXYLIC ACID

After distillation, pyrrole-2-carboxylic acid is developed in strongly acid solution with 4-dimethylaminobenzaldehyde.³¹³

Sub-Committee of the Analytical Methods Committee of the Society for Analytical Chemistry, *Analyst* **89**, 505-9 (1964).

³¹² Sidney Udenfriend and Perola Zaltsman, *Anal. Biochem.* **3**, 49-59 (1962).

³¹³ G. B. Gerber, K. R. Traeines, D. Wood and K. I. Altman, *Clin. Chim. Acta* **9**, 185-7 (1964).

Procedure—Urine. Acidify 15 ml. of urine with 0.8 ml. of concentrated hydrochloric acid. Distil with a short condenser to give 10 ml. of distillate. To 2.3 ml. of distillate, add 0.65 ml. of 2.5% solution of 4-dimethylaminobenzaldehyde in 95% ethanol and 0.35 ml. of concentrated hydrochloric acid. Exactly 5 minutes later, read at 560 m μ .

RIBOFURANOSIDES

The vicinal glycol groups in adenosine, adenosine-5-phosphoric acid, guanosine, cytidine, and uridine are determined by periodate consumption.³¹⁴ Iodate, unconjugated carbonyl, and carboxyl groups do not interfere.

Procedure—Fill 4 quartz absorption cells with a 1.00-cm. light path with water, and read, using cell No. 1 as a blank. Use a wave length as indicated in Table 34. Apply any absorbance difference found for the

TABLE 34. OPTIMUM ABSORPTION OF
RIBOFURANOSIDES

<i>Compound</i>	<i>Wave length, mμ</i>
Adenosine	227
Adenosine-5-phosphoric acid	227
Guanosine	224
Cytidine	227
Uridine	230

cells later as a correction. Empty cells No. 2, 3, and 4, dry, and fill as follows: add equal and known volumes of the sample to cells 2 and 4, and add an equal volume of water to cell 3. Add equal and known volumes of 0.002% sodium metaperiodate solution at approximately 20-100% in excess to cells 3 and 4. Add an equal volume of water to cell 2. Mix, and read at 5-10 minute intervals against cell 1 as a blank. The absorbance of cell 4 subtracted from the sum of the absorbancies of cells 2 and 3 represents the decrease in absorbance due to the consumption of periodate by the sample.

When the absorbance is constant, in 60-90 minutes, the reaction is complete. This difference divided by the absorbance of cell 3 at zero time is equal to the fraction of the known amount of added periodate that is consumed in the oxidation of the vicinal glycol groups.

³¹⁴ Jonathan S. Dixon and David Lipkin, *Anal. Chem.* 26, 1092-3 (1954).

PHENOTHIAZINE, THIODIPHENYLAMINE

Oxidation of phenothiazine by bromine in an ethanol medium produces red 3,7-dihydroxyphenazathionium bromide.³¹⁵ *p*-*tert*-Butyl catechol, Resin 731 SA, disproportionated resin, Lomar PW, and 2-anthraquinonesulfonic acid in 50 ppm. quantities do not interfere. Phenothiazine follows Beer's law for 0.002-0.1 mg. Phenothiazine and its oxidation products, phenothiazone, thionol, and phenothiazine-5-oxide, are separated by paper chromatography and thereafter read at, respectively, 480, 440, 590, and 520 m μ .³¹⁶ *N*-Substituted phenothiazines are extracted from alkaline solution with ether and read at 253 m μ .³¹⁷

Oxidation by hydrogen peroxide in 1:1 acetic acid gives a stable fluorescence with 31 phenothiazines and 3 thioxanthenes.³¹⁸ Phenothiazine in ethanol is treated with nitrite and acetic acid for reading.³¹⁹ Beer's law is followed up to 0.01 mg. per ml.

Phenothiazine is determined by reaction with *p*-dimethylaminobenzaldehyde.³²⁰ Accuracy is to $\pm 5\%$. Phenothiazine is separated on a chromatographic column of acid alumina and eluted with ether-petroleum ether for reading at 254-5 m μ .³²¹ Alternatively, to isolate, apply a solution of phenothiazine to a column of Celite 545 impregnated with the lower phase of 1:10 methylecyanide-hexane. Elute with the upper phase of the same mixture and read 10-ml. successive fractions at 253 m μ .³²² Carbazole, diphenylamine, and phenothiazine *S*-oxide do not interfere.

Procedure—*By bromine. Paper treated with elastomers.* Weigh a sample containing approximately 0.05 mg. of phenothiazine. Extract with two 10-ml. portions of ethyl ether. Evaporate the ether from the combined extracts at 100°. Add 10 ml. of 95% ethanol and heat for 10 minutes at 70°. Add 5 ml. of a saturated aqueous solution of bromine and gently agitate. Let stand at 70° for 15-20 minutes. Add an additional 5-ml. portion of the bromine solution, agitate, and let stand 15-20 minutes

³¹⁵ Kalman Marcali, *Anal. Chem.* **27**, 1586-94 (1955).

³¹⁶ W. T. Oliver, H. S. Funnell and N. Platonow, *J. Agr. Food Chem.* **9**, 213-14 (1961).

³¹⁷ J. Budinský, R. Zahradník and M. Chvapil, *Českosl. farm.* **9**, 299-303 (1960).

³¹⁸ J. B. Ragland and V. J. Kinross-Wright, *Anal. Chem.* **36**, 1356-9 (1964).

³¹⁹ B. Özsöz, *Turk. Bull. Hyg. Exp. Biol.* **24**, 293-7 (1964).

³²⁰ V. Pelloni and M. Sterescu, *Rev. Chim.* (Bucharest) **11**, 346 (1960).

³²¹ A. Brierly and D. M. Langbridge, *Analyst* **86**, 709-13 (1961).

³²² A. Holbrook, F. S. Barlow and F. Bailey, *J. Pharm. Pharmacol.* **15**, (Suppl) 232-5T (1963).

until all traces of free bromine are eliminated. At this point, the presence of phenothiazine is indicated by a red color. Remove from the bath, cool to room temperature, and dilute to 25 ml. with 95% ethanol. Read at 525 m μ .

By dimethylaminobenzaldehyde. Dilute a sample containing 0.002-0.01 mg. of diphenylamine in 20% methanol to 2 ml. with 20% methanol. Add 2 ml. of 1:2.6 sulfuric acid and 4 ml. of 0.15% solution of dimethylaminobenzaldehyde in 1:1 sulfuric acid. Add 2 drops of 30% hydrogen peroxide. Read after 20 minutes at 570 m μ against water.

By nitrite. Treat 5 ml. of sample in 95% ethanol containing about 0.1 mg. of phenothiazine with 1 ml. of 2% sodium nitrite solution and 1 ml. of 1:20 acetic acid. Let stand for 15 minutes and dilute to 10 ml. with 95% ethanol. Read at 475 m μ .

CARPHENAZINE, 10-{3-[4-(2-HYDROXYETHYL) PIPERAZIN-1-YL]
PROPYL}-2-PROPIONYLPHENOTHIAZINE, AS THE MALEATE

Determination of carphenazine involves acid reduction and direct reading.³²³

Procedure—Dissolve the solid or tablets in 50% ethanol to contain about 0.2 mg. of carphenazine per ml. As a reagent, mix 75 ml. of 0.1% palladous chloride solution and 44 ml. of 1:10 hydrochloric acid, and dilute to 500 ml. Mix 3 ml. of sample solution with 4 ml. of reagent and let stand for 10 minutes. Read at 490 m μ against a reagent blank.

5,6-DIHYDRO-5-(IMIDAZOLIN-2-YL-METHYL)-MORPHANTHIDINE

The method provides for separation of the test substance from acetyl derivatives of related compounds by thin-layer chromatography, and reading by fluorescence.³²⁴ Only the direct extraction is presented here.

Procedure—*Blood.* To 5 ml. of the sample, add 2 ml. of 0.8% sodium hydroxide solution and 20 ml. of ether. Shake for 2 minutes and centrifuge. Separate the ether and extract with four more 20-ml. portions of ether.

³²³ American Pharmaceutical Association Foundation, *J. Pharm. Sci.* 53, 101-3 (1964).

³²⁴ Frederick Tishler, H. E. Hagman, and S. M. Brody, *Anal. Chem.* 37, 906-10 (1965).

Evaporate the ether extracts in a stream of cold air. Transfer the residue to a 25-ml. flask with three 5-ml. portions of acetic anhydride. Heat at 100° for 45 minutes and dilute to 25 ml. with acetic anhydride. Excite a sample at 310 m μ and read the fluorescence at 410 m μ .

PROMETHAZINE, 10-(2-DIMETHYLAMINOPROPYL)PHENOTHIAZINE

Promethazine gives an appropriate color with ferrous ion for photometric reading.³²⁵ Beer's law is followed for 0.05-0.3 mg. per 25 ml. of final solution. With oxalic acid present, the complex is 1:3:3 ferric ion-promethazine-oxalic acid. A red complex with palladium is in a 1:1 ratio.

Procedure—Take a sample containing 0.1-0.3 mg. of promethazine. Add 1 ml. of 1:70 nitric acid and 1 ml. of 5% ferric ammonium sulfate solution. Dilute to 25 ml. and mix. Store in the dark for 24 hours. Read at 500 m μ against a blank.

GALANTHAMINE

Galanthamine in dilute sulfuric acid, when treated with potassium permanganate, gives a fluorescent reaction.³²⁶ The intensity is affected by the concentration of sulfuric acid, the amount of potassium permanganate, temperature, and the time of oxidation.

Procedure—To a 10-ml. sample containing 0.002-0.2 mg. of galanthamine in 1:175 sulfuric acid, add 2 ml. of 0.0316% potassium permanganate solution. Maintain at 5° \pm 1° for 2 hours. Decolorize with 2 ml. of 0.45% oxalic acid solution. Let stand overnight and read the fluorescence.

ϵ -CAPROLACTAM, 2-OXOHEXAMETHYLENIMINE

ϵ -Caprolactam is extracted from polymers, converted to the hydroxamic acid, and reacted with a ferric salt. The final color is red-violet and the range of determination is 0.002-15 mg. per ml. of sample.³²⁷ Beer's law is followed from 0.05-1.4 mg. ϵ -caprolactam.

³²⁵ I. Floderer and V. Horváthy, *Acta Pharm. Hung.* **32**, 193-8 (1962); P. Majlet and I. Bayer, *Acta Pharm. Hung.* **32**, 116-8 (1962).

³²⁶ Y. Ichimura and K. Nishimoto, *Bunseki Kagaku* **11**, 1024-7 (1962).

³²⁷ J. Horacek and Z. Malkus, *Z. anal. Chem.* **175**, 415-21 (1960); K. E. Istomina and L. A. Ionova, *Zavodskaya Lab.* **27**, 160-2 (1961); H. Mazur, *Koczni. Zakt. Hig. Warsaw* **14**, 509-16 (1963).

Caprolactam is read as the diazo compound before or after coupling with 1-naphthylamine.^{327a}

Procedure—*As hydroxamic acid.* Extract the sample with cold water for 24 hours or with boiling water for 2 hours. To a 1-ml. aliquot of the extract, add 1 ml. of 18% sodium hydroxide solution and 1 ml. of 8% aqueous hydrazine hemisulfate. Heat at 60° for 4.5 hours. Cool, and add 1 ml. of 2:3 hydrochloric acid. Add 1 ml. of 12% ferric chloride solution and dilute to 10 ml. with water. After 5 to 10 minutes, read at 500 m μ .

Alternatively, reflux a 50-ml. neutral solution of caprolactam for 30 minutes with 30 ml. of 21% hydroxylamine hydrochloride and 5 ml. of 4% sodium hydroxide solution. Cool to room temperature and acidify with 2.5 ml. of 1:5 hydrochloric acid. To the resulting hydroxamic acid, add 15 ml. of 10% ferric chloride solution and dilute to 100 ml. with water. Read at 500 m μ 10 minutes after the addition of ferric chloride.

By 1-naphthylamine. To 25 ml. of a sample containing more than 0.8 mg. of ϵ -caprolactam per ml. in 1:20 hydrochloric acid, add 1 ml. of 6.9% sodium nitrite solution. Mix, and after 20 minutes, add 1 ml. of 8.2% sulfamic acid solution. After 4 minutes, add 5 ml. of 0.5% solution of 1-naphthylamine solution in 1:20 hydrochloric acid. Keep at 40° for 2 hours and read the purple-red color.

As the diazo compound. To 15 ml. of sample in 1:20 hydrochloric acid containing 0.1-1 mg. of ϵ -caprolactam per ml., add 1 ml. of 6.9% sodium nitrite solution. Read the yellow color after 50 minutes at 20°.

MORESTAN, 6-METHYL-2,3-QUINOXALINEDITHIOL CYCLIC CARBONATE

Hydrolysis with ammonium hydroxide converts morestan to 6-methyl-2,3-quinoxalinedithiol. This gives a red complex with ammoniacal nickel solution.³²⁸ Purity of reagents and redistilled solvents are essential.

Procedure—*Apples and pears.* Chop the frozen sample, using 2 pounds of dry ice per pound of sample. Store frozen to let the dry ice sublime. Blend 200 grams with 400 ml. of acetone and blend 2 minutes longer. Filter, collecting 300 ml. of extract. Extract this with 300 ml. of light petroleum ether. Filter the extract through 250 grams of anhydrous

^{327a} V. Kalab and A. Hlavacova, *Chem. Prumyl.* **13**, 611-13 (1963).

³²⁸ R. Havens, J. M. Adams and C. A. Anderson, *J. Agr. Food Chem.* **12**, 247-8 (1964).

sodium sulfate. Wash the sodium sulfate with 50 ml. of petroleum ether. Evaporate the filtrate to 100 ml. on a steam bath, using an air jet. Filter, and wash the paper with 25 ml. and 25 ml. of solvent. Evaporate just to dryness.

Pour 100 ml. of benzene slurried with 30 grams of acid-washed alumina into a chromatographic column. Rinse down the sides with benzene. When the column has settled, cover with a pledget of glass wool.

Take up the dried sample with 15 ml. of benzene and add to the column. When this reaches the top of the column, add 15 ml. of the benzene used to rinse the beaker. Repeat with 10 ml. of benzene rinsings and follow with 75 ml. of benzene.

Evaporate the effluent to 25 ml. on a steam bath with an air jet and complete to dryness with the steam turned off. Take up the residue in 2 ml. of acetone and transfer with 7 ml. of concentrated ammonium hydroxide. Let stand for 10 minutes, shaking occasionally.

As a reagent, dissolve 6 grams of nickelous chloride hexahydrate in 50 ml. of water and add 50 ml. of concentrated ammonium hydroxide. Add 0.025 ml. of nickel reagent and shake occasionally for 20 minutes. Add 8 ml. of acetone and 3 ml. of benzene and shake. Repeat shakings until the aqueous phase is clear.

As a blank solution, mix 250 ml. of acetone, 175 ml. of concentrated ammonium hydroxide, and 0.625 ml. of the nickel reagent. Add 75 ml. of benzene to extract. Shake, and drain the aqueous layer.

Extract the benzene layer with 0.5 ml. of blank solution. Combine this and the aqueous phase. If red color remains in the benzene layer, extract with further 0.5-ml. portions of the blank solution. Dilute to 10 ml. with the blank solution and read at 540 m μ .

CHLOROQUINE

This is either a component of resotren or a chemical substance by itself. Chloroquine or its salts, pure or diluted with sodium chloride, is developed with cyanogen bromide and aniline.³²⁹ An alternative, applicable in the field, is to determine turbidimetrically with mercuric chloride and potassium iodide after extracting from urine with ether and from the ether layer with 1:175 sulfuric acid.³³⁰

³²⁹ Luiz Ribeiro Guimaraes and Emilia Pechnik, *Quimica* 37, 23-5 (1960).

³³⁰ Gerhard Fuhrmann, *Bull. World Health Organization* 22, 663-8 (1960).

Procedure—Alkalize an aqueous solution of the sample containing about 1 gram of chloroquine with ammonium hydroxide. Centrifuge off the free-base and wash the precipitate free of ammonia. Dry, and dissolve the residue in 95% ethanol. Dilute to 100 ml. with ethanol. To an aliquot containing a minimum of 0.2 mg. of chloroquine per ml., add 6 ml. of a 5.2% cyanogen bromide solution. Heat for 5 minutes at 70°, cool to 30°, and add 1 ml. of aniline. Dilute to 25 ml. with 95% ethanol. Let stand for 10 minutes in the dark to complete the reaction. Read at 420 m μ .

CHLORPROMAZINE, 2-CHLORO-10-(3-DIMETHYLAMINOPROPYL)-
PHENOTHIAZINE

The color developed for chlorpromazine in strong sulfuric acid with ferrous sulfate permits its determination.³³¹ The sulfoxalate and *N*-oxide of chlorpromazine also give the color, as do prochlorperazine, perphenazine, norechlorpromazine, thiopropazate, chlorprothixine, 2-hydroxypropromazine, promazine, triflupromazine, fluphenazine, and thioridazine.

Procedure—*Tissue*. Homogenize an appropriate sample with water. Make the homogenate alkaline with sodium hydroxide and extract with ether. Wash the ether layer with water. Extract the chlorpromazine from the ether layer with 1, 1, and 1 ml. of 1:17 sulfuric acid. Combine the acid extracts and drive off dissolved ether at 50-55°. Add 1 ml. of 2% ferrous ammonium sulfate solution and adjust the volume with 1:360 sulfuric acid. Cool in ice and add an equal volume of concentrated sulfuric acid. Let stand in the dark at room temperature for 30 minutes and read at 530 m μ .

PHENYLBUTAZONE, 4-BUTYL-1,2-DIPHENYL-3,5-PYRAZOLIDINEDIONE

Up to 0.25 mg. of phenylbutazone is determined with α,α' -bipyridine and ferric chloride,³³² or is read in the ultraviolet in alkaline solution.

Procedure—*General*. Dilute 1-ml. of a solution of the sample in absolute ethanol containing up to 0.25 mg. of phenylbutazone with 1 ml. of 0.25% ferric chloride solution in absolute ethanol and 1 ml. of 0.5% α,α' -bipyridine solution in absolute ethanol. Dilute to 25 ml. with absolute

³³¹ B. Gothelf and A. G. Karczmar, *Intern. J. Neuropathol.* 2, 95-9 (1963).

³³² Michel Deffner and Alice Issidorides-Deffner, *Chim. anal.* 40, 460-2 (1958).

ethanol and read after 20 minutes at 520 $m\mu$ against a reagent blank. Determine by the reduction in color.

Tablets. Crush the tablets and suspend in water. Extract the phenylbutazone from the suspension with benzene. If calcium phenylbutazone is present, acidify to pH 3 before extraction. Evaporate the benzene extracts to dryness and take up in an appropriate amount of 0.4% sodium hydroxide solution. Read at 264 $m\mu$.

1-PHENYL-2,3-DIMETHYL-4-BROMO-5-PYRAZOLONE

2-Naphthol is a reagent for 1-phenyl-2,3-dimethyl-4-bromo-5-pyrazolone in serum.³³³ For a 1-cm. cuvet, the concentration should be in the range of 0.5-8 mg. %.

Sample—Serum. To prepare the sodium acetate buffer, dissolve 118 grams of sodium acetate and 56 ml. of acetic acid in water and dilute to 1 liter. As a reagent, dissolve 20 grams of 2-naphthol in 150 ml. of 95% ethanol and dilute with 50 ml. of water. Heat 2 ml. of the sample solution with 1 ml. of the sodium acetate buffer and 2 ml. of water for 5 minutes at 100°. Filter, and wash with two 2-ml. portions of water. Add 1 ml. of concentrated hydrochloric acid and 2 ml. of bromine water to the filtrate and washings. Heat until colorless. Add 1 ml. of 2-naphthol reagent, cool, and add 3 ml. of concentrated ammonium hydroxide. After 10 minutes, extract with 3 ml. of chloroform and filter through paper. Dry the extract with calcium chloride and read at 500 $m\mu$.

1,2,3,4-TETRAHYDROAMINOACRIDINE

Methyl orange under suitable conditions forms complexes with several organic bases, including the above.³³⁴ With the methyl orange reagent, Beer's law is followed up to 0.05 mg. per ml. As little as 0.0005 mg. per ml. can be measured with 5% accuracy. In aqueous solution, tetrahydroaminoacridine shows an absorption spectrum in the ultraviolet with peaks at 323 and 335 $m\mu$ suitable for direct reading.

Procedure—Urine and other fluids. By methyl orange. The methyl orange reagent consists of equal parts of 0.5% methyl orange solution

³³³ Wolfgang Strunz, *Sci. Pharm.* **27**, 53-5 (1959).

³³⁴ B. B. Brodie and S. Udenfriend, *J. Biol. Chem.* **158**, 705-14 (1945); P. N. Kaul, *J. Pharm. Pharmacol.* **14**, 237-42 (1962).

and 3% boric acid solution at pH 5. Filter, and keep at 40° to prevent crystallization. To a 10-ml. sample containing 0.0002-0.003 mg. of tetrahydroaminoacridine per ml., add 0.2 ml. of a saturated sodium carbonate solution to produce a pH not lower than 10. Shake with 26 ml. of ethylene dichloride for 7 minutes on a mechanical shaker. Centrifuge at 2000 rpm for 5 minutes. Add a 25-ml. aliquot of the ethylene dichloride extract to 0.5 ml. of the methyl orange reagent. Shake mechanically for 7 minutes and centrifuge. Transfer a 24-ml. portion of the organic layer to 4 ml. of 1:19 hydrochloric acid. Shake mechanically for 5 minutes and read the acid layer at 508 $m\mu$ against 1:19 hydrochloric acid.

In the ultraviolet. Shake a 10-ml. sample for 7 minutes with 0.2 ml. of concentrated ammonium hydroxide solution and 23 ml. of chloroform. Centrifuge, and shake 20 ml. of the organic layer for 5 minutes with 5 ml. of 1:19 hydrochloric acid. Centrifuge, and read the acid layer at 323 $m\mu$ against 1:19 hydrochloric acid saturated with chloroform.

POLYVINYLPYRROLIDONE

In the determination of polyvinylpyrrolidone by iodine, the reaction has been found to involve free iodine and not the I_3^- ion.³³⁵ With reagents prepared from iodine in aqueous potassium iodide, the intensity of the color depends on the concentration of free iodine, the reaction of the colored complex of iodine with polyvinylpyrrolidone being similar to that with starch. Ethanol interferes. Sensitivity is improved by carrying out the reaction in citric acid to control the pH and eliminate partial precipitation of polyvinylpyrrolidone.³³⁶

The main disadvantage of the method is the variation of the absorbance with the molecular weight of the polyvinylpyrrolidone. To take this into account, the polyvinylpyrrolidone content of the sample should be read off the calibration curve corresponding to the K value of the sample. Beer's law is followed for 0.005-0.03 mg. per ml. An alternative method for polyvinylpyrrolidone is based on the color formed by reaction with iodine and zinc sulfate.³³⁷ Beer's law is followed for 0-22 mg. of polyvinylpyrrolidone per 100 ml. sample.

Two methods are based on the reaction of polyvinylpyrrolidone with

³³⁵ H. Campbell and G. Hunter, *Lancet* **264**, 197-8 (1953).

³³⁶ Gabor B. Levy and David Fergus, *Anal. Chem.* **25**, 1408-10 (1953).

³³⁷ C. Discombe and H. B. W. Creig, *Ann. biol. clin.* **12**, 415-18 (1954).

sulfuric acid.³³⁸ By one procedure, the polyvinylpyrrolidone is read after heating with sulfuric acid at 100°. In the second method, the polyvinylpyrrolidone is heated with carbazole in sulfuric acid.

The difference between the optical density of Brilliant Vital Red dye in water and the optical density of the dye and polyvinylpyrrolidone in water is roughly proportional to the concentration of polyvinylpyrrolidone present.³³⁹ Most of the uncombined dye is removed from the aqueous phase with *n*-butanol. However, since the unbound dye is not completely removed by *n*-butanol, it is necessary to subtract the optical density reading obtained with the filtrate of a sample containing no polyvinylpyrrolidone from readings obtained with standards and unknowns. The standards must be run at the same time as the sample. Over 0.01 mg. of polyvinylpyrrolidone can be measured.

Procedure—*By potassium triiodide. Serum.*³⁴⁰ As a reagent, mix 67 mg. of potassium iodide and 33 mg. of iodine in 100 ml. of water. Add 5 ml. of 10% trichloroacetic acid solution to a 1-ml. sample. Shake and let stand at room temperature for 5 minutes. Filter. To 5 ml. of the filtrate, add 1 ml. of iodine reagent and let stand for 20-25 minutes. Read at 520 m μ .

Serum or plasma. To a 2-ml. sample, add 4 ml. of 38% citric acid solution and 12 ml. of water. Add 2 ml. of 7% sodium tungstate solution. Shake, and let stand for 2-5 minutes. Filter, and take an aliquot containing 0.005-0.03 mg. of polyvinylpyrrolidone per ml. To prepare the potassium triiodine solution, dissolve 0.81 gram of sublimed iodine and 1.44 grams of potassium iodide per liter of water. To a 10-ml. aliquot of the sample, add 2 ml. of potassium triiodide reagent. Swirl, and transfer immediately to a cuvet. Read at 500 m μ against water. In warm weather, cool the reagent, sample, and spectrophotometer compartment to 20°.

Aqueous or saline solution or urine. Dilute the sample with 7.6% citric acid so that the concentration of polyvinylpyrrolidone is 0.005-0.03 mg. per ml. Develop as for serum or plasma, from "To a 10-ml. aliquot of the sample . . ." Since some urine samples have a bleaching effect on the polyvinylpyrrolidone complex, dilute these samples further than usual, although there is a decreased precision.

³³⁸ Luigi Costabile, Pasquale Leonetti and Enrico Sanseverino, *Biochim. appl.* 3, 81-6 (1956).

³³⁹ Francis P. Chinard, *J. Lab. Clin. Med.* 39, 666-72 (1952).

³⁴⁰ Guido Moricca, *Rass. med. sper.* 2, 65-9 (1955).

By iodine and zinc sulfate. Plasma. To an 8-ml. sample, add 0.5 ml. of 0.6 saturated cupric sulfate pentahydrate solution and 0.5 ml. of 0.6 saturated potassium ferrocyanide. Mix and centrifuge. To prepare the reagent, mix 5 grams of zinc sulfate heptahydrate and 10 ml. of a 1.3% solution of iodine in 3.3% potassium iodide solution, and dilute to 100 ml. with water. Dilute a 5-ml. portion of the plasma sample with 5 ml. of water. To this sample, containing up to 0.11 mg. of polyvinylpyrrolidone, add 1 ml. of reagent and read at 432 $m\mu$ against a reagent blank.

By carbazole in sulfuric acid. Biological fluids. To a 2-ml. sample, add 2 ml. of 5% trichloroacetic acid solution containing 0.1% of silver sulfate and let stand for several hours. Centrifuge for 20-30 minutes and mix the liquid with an equal volume of 40% trichloroacetic acid solution. Centrifuge for 10 minutes, add 1 ml. of water to the precipitate, and dissolve the precipitate in saturated sodium hydroxide solution. To prepare the carbazole reagent, heat 5 ml. of concentrated sulfuric acid and 1 ml. of water to 120-130°. Add 0.3 ml. of 0.5% carbazole solution in absolute ethanol. Cool to 0° and adjust the volume with concentrated sulfuric acid. To a 1-ml. sample containing 0.937-60 mg. of polyvinylpyrrolidone per ml., add 5 ml. of carbazole reagent. Shake, and heat at 100° for 10 minutes. Cool, and read at 500 $m\mu$.

By brilliant vital red. Serum or plasma. As a protein precipitant, dissolve 25 grams of zinc sulfate heptahydrate in 250 ml. of 1:143 sulfuric acid and dilute to 1 liter with water. Twenty-five ml. of this solution should be equivalent to 6.75 ml. or slightly less of 3% sodium hydroxide solution using the phenolphthalein end point. To remove the protein, add 1.25 ml. of water, 0.6 ml. of protein precipitant, and 0.15 ml. of 3% sodium hydroxide solution to a 0.25-ml. sample. Let stand for 20 minutes with occasional stirring. Centrifuge.

To prepare the phosphate buffer at pH 7, dissolve 117.77 grams of dibasic potassium phosphate and 44.11 grams of monobasic potassium phosphate in water and dilute to 1 liter. To 0.5 ml. of the sample add 1 ml. of water, or to 1 ml. of the supernatant layer, add 0.5 ml. of water. Add 1.5 ml. of phosphate buffer and 0.2 ml. of 0.05% Brilliant Vital Red solution. Mix thoroughly and let stand for 45 minutes. Add 3 ml. of *n*-butanol. Stopper with rubber stoppers and place in a rocking device. Motion should be no more violent than that which is used for mixing blood before taking samples for hematocrit estimations. Such a device as is illustrated in Figure 13 is necessary to ensure identical treatment of

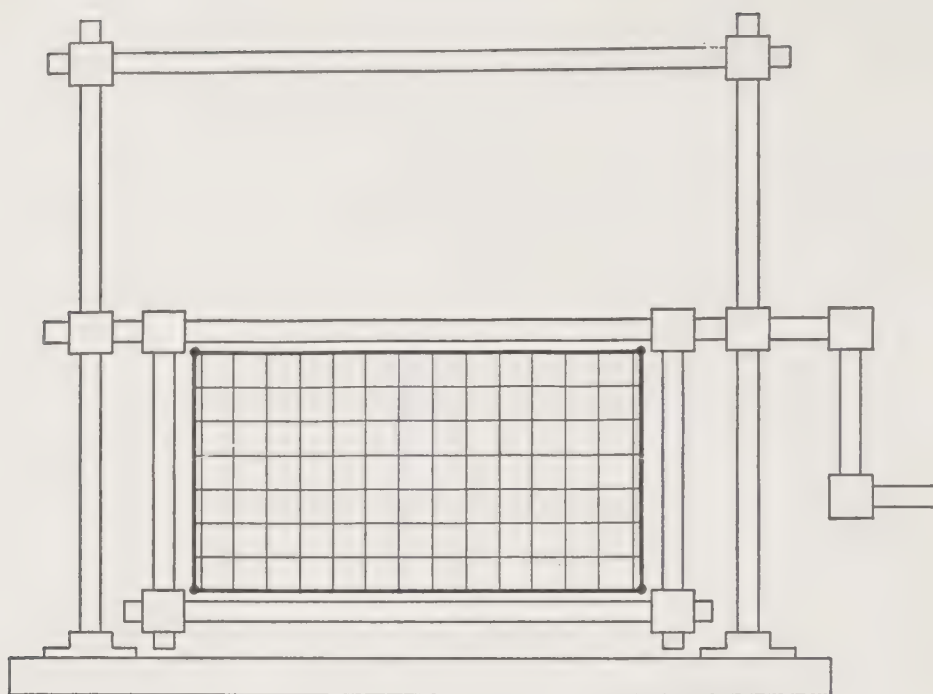


FIG. 13.
Rocker for determination of polyvinylpyrrolidone

standards and unknowns. This particular device is made of Fisher Flexa-frame parts. The wire test tube rack is held in place with rubber bands. A sheet metal plate of an area equal to that of the rack shown is placed over the tubes and is also attached with rubber bands. The movement used is about 35 degrees away from the vertical rest position in each direction. The rocking should be sufficiently slow so that the liquid phases travel from one end of the tube to the other and so that emulsion formulation is kept to a minimum.

Rock gently for 15 minutes. Remove the stoppers and cover the tubes with parafilm. Centrifuge for 30 minutes. Pipet out the lower aqueous layer, leaving any white precipitate at the bottom of the tubes undisturbed. Seal with parafilm. Read. The wave length of maximum difference in optical density between the blank and the sample should be determined for each instrument. One wave length that has been used is 510 $m\mu$.

Whole blood. Prepare a protein precipitant as described under "serum or plasma." To remove the protein in a 0.2-ml. sample, add 1 ml. of water, 0.8 ml. of protein precipitant, and 0.2 ml. of 3% sodium hydroxide

solution. Let stand for 20 minutes, stirring occasionally. Centrifuge. Complete as described for serum or plasma, starting at "To prepare the phosphate buffer . . ."

By sulfuric acid. To a 1-ml. sample containing 0.1-60 mg. of polyvinylpyrrolidone, add 1 ml. of concentrated sulfuric acid and heat at 100° for 30 minutes. Cool, and read at 500 $m\mu$.

2-METHYL-3-*o*-TOLYL-4(3H)QUINAZOLINONE

2-Methyl-3-*o*-tolyl-4(3H)quinazolinone is extracted from plasma into hexane. After evaporation of the hexane, the quinazolinone is dissolved in hydrochloric acid and read in the ultraviolet.³⁴¹ The method is specific for 2-methyl-3-*o*-tolyl-4(3H)quinazolinone and as low as 0.001 mg. per ml. of final solution can be measured.

Procedure—Plasma. To a 2-ml. sample, add 3 ml. of 1:110 hydrochloric acid, 1 ml. of 4% sodium hydroxide solution, and 30 ml. of hexane. Shake for 60 minutes and centrifuge at 3000 rpm for 10 minutes. Evaporate 20 ml. of the organic phase to dryness *in vacuo* at 50°. Dissolve the residue in 5 ml. of 1:110 hydrochloric acid. Read at 234 $m\mu$ against a reagent blank.

Urine. Follow the procedure for plasma and read at 270 $m\mu$.

Tissue extracts. Follow the procedure for plasma and read at 260 $m\mu$. For tissues, it is preferable to use a triple-point method at 234, 255, and 285 $m\mu$.

2-ANILINOMETHYL-2-IMIDAZOLINE HYDROCHLORIDE

Treatment of 2-anilinomethyl-2-imidazoline hydrochloride with sodium nitroprusside in alkaline solution produces a violet color that follows Beer's law for up to 0.5 mg. per ml.³⁴² An excess of sodium bicarbonate is required for full development of the color, which is stable for 30 minutes. Aldehydes and certain other compounds that react with the sodium nitroprusside reagent interfere, but they are usually not found in pharma-

³⁴¹ Masuo Akagi, Yoneshiro Oketani and Masahiko Takada, *Chem. Pharm. Bull.* **11**, 62-7 (1963).

³⁴² S. C. Slack and W. J. Mader, *J. Am. Pharm. Assoc.* **46**, 742-4 (1957).

ceutical preparations with the imidazoline. This procedure is also applicable to antazoline hydrochloride, tolazoline hydrochloride, and naphazoline hydrochloride.

Procedure—Dilute a 1-ml. sample so that the concentration of 2-anilinomethyl-2-imidazoline hydrochloride is equivalent to 25 mg. per 100 ml. In the case of antazoline hydrochloride and of naphazoline hydrochloride, dilute so that the sample is equivalent to 50 mg. per 100 ml.

To a 1-ml. sample, add 0.5 ml. of 4% sodium hydroxide solution and 0.5 ml. of 5% sodium nitroprusside solution. Mix well and let stand for 10 minutes. Add 1 ml. of 8.4% sodium bicarbonate solution and dilute to 10 ml. with water. Mix, and let stand for 10 minutes. Read against a reagent blank at 565 m μ .

ANTAZOLINE, 2-(N-BENZYLANOLINOMETHYL)-2-IMIDAZOLINE

For determination by sodium nitroprusside, see 2-anilinomethyl-2-imidazoline hydrochloride.

TOLAZOLINE, PRISCOLINE, 2-BENZYL-2-IMIDAZOLINE

For determination by sodium nitroprusside see 2-anilinomethyl-2-imidazoline hydrochloride.

PRIVINE, NAPHAZOLINE, 2-(1-NAPHTHYLMETHYL)IMIDAZOLINE

Naphazoline is determined by an acid-dye method.³⁴³ Hydrolytic products do not interfere. This is more rapid than the National Formulary method and more specific than the usual spectrophotometric one. An ultraviolet method is also available.³⁴⁴ To determine naphthazoline hydrochloride, in the ultraviolet, make alkaline and extract with chloroform. Evaporate the extract, take up in 1:100 hydrochloric acid, and read at 281 m μ . The method is applicable to 0.005-0.03 mg. per ml. For determination by sodium nitroprusside, see 2-anilinomethyl-2-imidazoline hydrochloride, page 581.

HYPOXANTHINE, 6(1H)-PURINONE

For hypoxanthine, see purines, page 560.

³⁴³ N. Mattson and R. N. Gaebeler, *Drug Std.* **28**, 77-8 (1960).

³⁴⁴ J. Kracmarova, *et al.*, *Cesk. farm.* **10**, 83-5 (1961).

INOSINE, HYPOXANTHINE RIBOSIDE

For inosine, see purines, page 560.

HYPOTAURINE

Hypotaurine is oxidized by a sodium hydroxide-iodine solution. The excess of hypoiodite is converted to iodine and the iodine liberated is developed with starch and read at 590 m μ .³⁴⁵ The procedure is applicable for 5-40 micromoles of hypotaurine per ml. The following ions do not interfere: magnesium, cadmium, aluminum, zinc, and molybdate. The following ions increase color development: manganese, vanadate, cobalt, and nickel. Addition of ethylenediaminetetraacetic acid does not remove the effects of these interfering ions. The following deproteinizing agents have no effect: *M* perchloric acid, *M* metaphosphate, 0.08 *M* uranium acetate, and Somogyi's zinc sulfate deproteinizing agent. Lead acetate causes precipitation of lead iodide. Protein concentration over 0.003 mg. per ml. interferes. The interference of trichloroacetic acid depends on the concentration of hypotaurine. The following also interfere: sulfuric oxide, cysteine, cysteine sulfinic acid, cysteamine, formaldehyde, acetaldehyde, and acetone. Volatile compounds such as acetone, formaldehyde, and acetaldehyde can be removed by microdiffusion or by evaporation under reduced pressure.

Procedure—Mix 1 ml. of 4% sodium hydroxide solution, 0.1 ml. of iodine solution containing 0.5 mg. per ml., and 1 ml. of 16.6% potassium iodide solution. Add an appropriate volume of the sample containing hypotaurine. Let stand at room temperature for 5 minutes and add 1 ml. of 0.2% starch solution. Dilute the blue solution to 10 ml. with water and read at 590 m μ .

CAPTAN, *N*-(TRICHLOROMETHYLTIO)-3a,4,7,7a-TETRAHYDROPHthalimide

When captan is fused with resorcinol at 135°, a red color develops.³⁴⁶ Upon addition of glacial acetic acid, a yellow color is formed.³⁴⁷ The captan is stripped from the sample with benzene. Water waxes and color

³⁴⁵ Koichiro Sumizu, *Anal. Biochem.* **4**, 378-83 (1962).

³⁴⁶ Allen Kittleson, *Anal. Chem.* **24**, 1173-5 (1952).

³⁴⁷ Donald M. Taylor, *J. Assoc. Offic. Agr. Chemists* **40**, 219-24 (1957); T. J. Klayder, *ibid.* **46**, 241-2 (1963).

are removed from the benzene extract. Beer's law is followed up to 0.15 mg.

Procedure—*Fruits and vegetables.* The resorcinol must be free of discoloration and pass the following tests: Fuse 0.5 gram and dissolve in 25 ml. of glacial acetic acid. The absorbance at 425 m μ is not more than 0.015 against acetic acid. One gram should not lose more than 2 mg. in 4 hours over sulfuric acid. If more is lost, dry over sulfuric acid until the test is satisfactory. The mixture for removal of water, color, and waxes contains 10 parts of Nuchar, 5 parts of Hyfluosuperflo, and 5 parts of anhydrous sodium sulfate.

To a 500-gram sample in a jar with a solvent-tight lid, add 500 ml. of benzene. Agitate for 15 minutes and transfer the benzene to a separatory funnel. If there is no separable aqueous layer, this step may be omitted. Decolorize and dehydrate a 100-ml. portion of the benzene extract with 3-4 grams of the mixture to remove water, color, and waxes. Shake vigorously for 5 minutes and filter through a folded paper, discarding the first 10-15 ml. of filtrate. To 5 ml. of the filtrate, add 0.5 gram of resorcinol. Heat in an oil bath at 135° for 20 minutes, immersing the reaction tubes to a depth of 2 inches, but not allowing them to touch the bottom of the bath. A brilliant red color develops. Remove from bath and immediately add 10-15 ml. of glacial acetic acid. Rapidly immerse in water at room temperature. Dilute to 25 ml. with glacial acetic acid. Read at 425 m μ within 1 hour against acetic acid.

QUINALDIC ACID

See Kynurenic acid, page 555.

4-QUINOLONE

See Kynurenic acid, page 555.

N-METHYL-4-QUINOLONE

See Kynurenic acid, page 555.

ETHOXYQUIN, 1,2-DIHYDRO-6-ETHOXY-2,3,4-TRIMETHYLQUINOLINE

Ethoxyquin has been determined by its fluorescence,⁸⁴⁸ but this

⁸⁴⁸ E. M. Bickoff, J. Guggolz, A. L. Livingston and C. R. Thompson. *Anal. Chem.* 28, 376-S (1956).

changes with storage conditions. Also, other antioxidants fluoresce. Ethoxyquin is readily extracted from a hydrocarbon solution with dilute hydrochloric acid and read in the ultraviolet. The only interfering antioxidant is butylated hydroxyanisole. If present, it is saponified and left behind as an alkali salt in the extraction with hydrocarbon.³⁴⁹

Procedure—Butylated hydroxyanisole absent. *Solids.* Reflux a sample containing 4-15 mg. of ethoxyquin with 40 ml. of heptane for 20-30 minutes. This extracts fats, vitamins, etc., as well as ethoxyquin. Shake with an equal volume of 1:20 hydrochloric acid. Separate the acid layer and read at 286 m μ .

Liquids. Dissolve in the heptane, and without refluxing, extract with acid.

Butylated hydroxyanisole present. Reflux a solid or liquid sample containing 4-15 mg. of ethoxyquin with 50 ml. of 50% ethanol for 20 minutes. Add 10 ml. of 40% potassium hydroxide solution and continue to reflux for 10 minutes. All fatty materials and the BHA are saponified. Extract the ethoxyquin with 35, 35, and 25 ml. of heptane. The BHA remains behind as the potassium salt. Wash the combined hydrocarbon extracts with water until neutral to phenolphthalein. Extract with 45, 45, 45, and 45 ml. of 1:20 hydrochloric acid. Combine the acid layer and read at 286 m μ .

XANTHURENIC ACID, 4,8-DIHYDROXYQUINALDIC ACID

For separation from related tryptophan metabolites and development with diazotized sulfanilic acid, see kynurenine, page 294. Xanthurenic acid is coupled with *p*-diazobenzenesulfonic acid in a borate buffer for pH 7.09.³⁵⁰ This is read at 496 m μ .

XANTHURENIC ACID 8-METHYL ETHER

For separation from related tryptophan metabolites and development by fluorescence, see kynurenine, page 294.

³⁴⁹ Thomas Choy, N. J. Aleino, H. C. Klein and J. U. Quattrone, Jr., *J. Agric. Food Chem.* **11**, 340-2 (1963).

³⁵⁰ M. Kaleb, *Acta Univ. Polackienae Olemuc.* **23**, 125-32 (1961).

TROPACIN, TROPINEDIPHENYL ACETATE

Tropacine is the hydrochloride of the tropinal ester of phenylacetic acid. It is determined nephelometrically by treatment with sodium phosphotungstate.³⁵¹

Procedure—Dissolve the sample in water and dilute to 50 ml. Take a 0.8-ml. aliquot when the amount of tropacine is 0.015 gram, or a 1.2 ml. aliquot when the amount of tropacine is 0.01 gram. Add 20-30 ml. of water and 1 ml. of 0.01 *M* sodium phosphotungstate solution. Dilute to 50 ml. and mix. Determine the turbidity nephelometrically.

PALFIUM, R875, 4-[2-METHYL-4-Oxo-3,3-DIPHENYL-4-(1-PYRROLIDINYL)BUTYL] MORPHOLINE

Palfium after a complex separation is read as the hydrochloride.³⁵²

Procedure—*Biological media.* Macerate with 60% ethanol acidified with tartaric acid and digest for 12 hours at 60°. Concentrate, and add absolute ethanol to precipitate proteins. Filter, evaporate the filtrate, and take it up in hot 1:100 hydrochloric acid. Cool, and extract with alkaline 1:1 chloroform-ether. Evaporate the extract and take up in 60% ethanol acidified with tartaric acid. Filter and evaporate to dryness. Take up the residue in chloroform, filter, and evaporate to dryness. Take up in isopropanol acidified with hydrochloric acid and read at 248-265 $m\mu$.

VITAMIN B₁₂, CYANOCOBALAMIN

Vitamin B₁₂ may be read directly in benzyl alcohol after treatment with cyanide. Alternatively, a portion of the solution may be converted to dicyanocobalamin and the difference in absorbance between cyanocobalamin and dicyanocobalamin read at 582 $m\mu$,³⁵³ (cf. Vol. IV, pp. 315-16). The method is suitable for broths and cultures. For application

³⁵¹ G. I. Luk'yanchikova, *Med. Prom. SSSR* **12**, 35-8 (1958).

³⁵² M. Attisso, *Thérapie* **14**, 650-60 (1959).

³⁵³ G. O. Rudkin, Jr. and R. J. Taylor, *Anal. Chem.* **24**, 1155-6 (1952); Robert A. Fisher, *J. Agr. Food. Chem.* **1**, 951-3 (1953); Hans Kurmeier, *Die Fischwirtschaft* **6**, 46-8 (1954); M. Covello and O. Schettino, *Ann. Chim., Roma* **52**, 1135-46 (1962).

to liver extracts, first extract with benzyl alcohol, then with butanol to eliminate interfering impurities.³⁵⁴ Riboflavin is removed by Amberlite IRA-400 or by strongly basic Zerolite F.F.

Vitamin B₁₂ is also oxidized by hydrogen peroxide and the 4% of cobalt coupled with nitroso-R salt for reading at 420 m μ .³⁵⁵ Color from metals other than cobalt can be destroyed by nitric acid. Ionic cobalt and iron in the vitamin may be removed by sorbing vitamin B₁₂ on a column of Amberlite CG50.³⁵⁶ Washing with acetone removes vitamins A, D, and E. Then elution with dioxan and hydrochloric acid recovers the cobalamine. Dry cellular samples are extracted with benzyl alcohol; moist samples require *n*-propanol to extract vitamin B₁₂. For total cobalamines in acetate buffer for pH 5.5, reading is at 351 m μ .³⁵⁷ Homologs of vitamin B₁₂ that contain cobalt interfere. The error is $\pm 6\%$. Vitamin B₁₂ can be determined by its cyanide group.³⁵⁸

Vitamin B₁₂ is read in aqueous solution at 361 m μ ,³⁵⁹ 365 m μ ,³⁶⁰ or 580 m μ .³⁶¹ Thus, cyanocobalamin and uridine-5¹-triphosphate are separated from pharmaceutical preparations on an anion-exchange column. Then the vitamin B₁₂ is read at 361 mg. and uridine-5¹-triphosphate at 262 mg.³⁶² Loss of vitamin B₁₂ by coprecipitation with protein from serum is greatly reduced by addition of 0.002 mg. of potassium cyanide per ml.

The spectrophotometric method does not differentiate between vitamin B₁₂ and pseudovitamin B₁₂.³⁶³ A complex of the cobalt in cyanocobalamin with Fast Navy 2R is formed at pH 8.5 and read at 620 m μ .³⁶⁴ In the

³⁵⁴ Aparicio Dominguez, Gayo Oller and Martinez Oller, *Galenica Acta* (Madrid) **14**, 157-63 (1961).

³⁵⁵ R. K. Mitra, P. C. Bose, G. K. Ray and B. Mukerji, *Indian J. Pharm.* **24**, 152-4 (1962).

³⁵⁶ D. Monnier and Y. Ghaliounghi, *Chimia* **16**, 340 (1962); Mohammad Abul Hussain Sharif, *Pakistan J. Sci. Ind. Research* **1**, 160-4 (1958); D. Monnier, Y. Ghaliounghi and R. Saba, *Anal. Chim. Acta* **28**, 30-40 (1963).

³⁵⁷ C. Cardini, G. Cavina, E. Cingolani, A. Mariani and C. Vicari, *Farmaco, Ed. Prat.* **17**, 583-92 (1962).

³⁵⁸ D. Monnier, R. Saba and Y. Galiounghi, *Helv. Chim. Acta* **46**, 2558-65 (1963).

³⁵⁹ Ryszard Kojer, *Acta Polon. Pharm.* **15**, 359-69 (1957).

³⁶⁰ Enrique Jedinger Hauser, *Anales fac. quim. farm., Univ. Chile* **8**, 364-74 (1956).

³⁶¹ Bernardo M. Oller, Nemesio Gayo Fernandez and Luis Aparicio Dominguez, *Arch. inst. farmacol. exptl.* (Madrid) **8**, 55-60 (1956).

³⁶² M. Marini-Scotti, *Farmaco, Ed. Prat.*, **18**, 332-4 (1963).

³⁶³ Louis Chalet, Thomas Miller and A. E. Boley, *J. Agr. Food Chem.* **2**, 784-6 (1954).

³⁶⁴ A. A. Abd El Raheem and M. M. Dikhana, *Z. anal. Chem.* **189**, 389-96 (1962).

presence of EDTA, the organic part of the molecule does not interfere. Differential spectrophotometry at 361 $m\mu$ is very accurate.³⁶⁵

Procedure—By added cyanide. General. Take a sample of vitamin B₁₂ solution containing about 200 micrograms in not more than 200 ml. Under a hood, make the solution to 1% with sodium cyanide. Adjust the pH to 9.5-10 with 10% sodium hydroxide solution. In 5 hours, standing at room temperature, the vitamin B₁₂ will all be present as the dicyanide complex.

Dissolve 20% of solid sodium sulfate in the treated sample. Adjust the pH to 11-11.5 with 10% sodium hydroxide solution. Extract with three one-tenth volumes of benzyl alcohol. Combine the benzyl alcohol extracts and add one-half volume of chloroform. Extract with three one-tenth volumes of water. Dilute the combined aqueous extracts to 25 ml. To one 10-ml. aliquot add 2 ml. of 10% sodium cyanide solution. Adjust the pH of another 10-ml. aliquot to 5-6 by adding 2 ml. of 12.5% solution of potassium dihydrogen phosphate. Read each at 582 $m\mu$ and use the difference for determination.

Dry bacteria cells. The sample desirably contains at least 0.1 mg. of vitamin B₁₂. If less than 0.01 gram is present in a 10-gram sample, supplement with standard vitamin B₁₂. If the sample is alkaline or poorly buffered, neutralize 5 ml. of 1% sodium cyanide solution with 2:3 hydrochloric acid in a well-ventilated hood. Add 100 ml. of benzyl alcohol and mix. Pipet 50 ml. of the benzyl alcohol solution into the sample. Mark the volume of the solution on the tube.

Heat at 100° so that the temperature of the sample is at least 92° for 15 minutes, stirring frequently. Cool to room temperature and add the benzyl alcohol solution to the marked volume on the tube. If the suspension gels on cooling, reheat, and centrifuge while hot for 5-10 minutes. Then decant and measure the supernatant layer. To a 10-ml. portion of the treated sample, add 5 ml. of 95% ethanol containing 6 ml. of 10% sodium cyanide solution per 100 ml. To another 10 ml. portion, add 5 ml. of 95% ethanol containing 6 ml. of 10% citric acid per 100 ml. Mix, and filter if necessary. Read the difference in absorbance at 582 $m\mu$. Calculate as follows:

Vitamin B₁₂ in gammas per gram of sample

$$= (\Delta A \times 1.5 \times 50) / (0.0054 \times \text{sample weight})$$

in which ΔA = the difference in absorbance.

³⁶⁵ J. Bayer, *Pharmazie* 19, 602-5 (1964).

If vitamin B₁₂ has been added to the sample, add the volume used to the factor 50 and subtract the B₁₂ added per gram of sample from the result.

To read directly, measure the sample in benzyl alcohol-cyanide solvent and add, while still warm, half its volume of chloroform. Extract with 8-ml., 7-ml., and 7-ml. portions of water, allowing 10 minutes for each extraction. Dilute the combined water extracts to 25 ml. with water and let stand for 5 minutes. To a 10-ml. aliquot of the extract, add 2 ml. of 10% sodium cyanide solution. To another 10 ml. aliquot, add 2 ml. of 12.5% potassium dihydrogen phosphate solution. Mix, and let stand for 30 minutes. Read at 582 m μ . Calculate as follows:

Vitamin B₁₂ in gammas per gram of sample = $(\Delta A_s 6/5 \times 25 \times 1.03 \times 50)/(0.0054 \times \text{vol. of supernatant liquid} \times \text{sample weight})$
in which ΔA_s = the absorbance difference.

Moist cellular material. Centrifuge a sample containing up to 3 grams of solids in a borosilicate tube and discard the supernatant liquid. Mix 100 ml. of *n*-propanol with 10 ml. of 0.5% sodium cyanide solution. Add 40 ml. of the propanol solution to the sample. If the sample contains less than 0.01 mg. of vitamin B₁₂ per gram of solids, add B₁₂ standard solution to make the total content at least 0.1 mg. Mark the volume on the tube. Heat at 100° for 30 minutes so that the suspension boils gently for at least 15 minutes, stirring frequently. Cool to room temperature. Restore to the original marked volume with the propanol solution. Centrifuge for 10 minutes.

To a 10-ml. aliquot of the supernatant liquid, add 2 ml. of 10% sodium cyanide solution. To another 10-ml. aliquot, add 2 ml. of 5% citric acid solution. Mix gently, and let stand for 30 minutes. Filter if necessary, and measure the difference in absorbance at 582 m μ .

Vitamin B₁₂ in gammas per gram of sample
= $(\Delta A \times 11.9/10 \times \text{total vol. as marked on tube})/0.0054$
in which A = the absorbance difference.

Liver. To an extract of the sample containing 0.1 mg. of vitamin B₁₂, add 1% by weight of crystalline sodium cyanide. Let stand for 5 hours at pH 10. Add sodium sulfate to a concentration of 20% and extract with three 5-ml. portions of benzyl alcohol. Mix the combined extracts with 7.5 ml. of chloroform and extract three times with a total of 12.5 ml. of water. Dilute the combined aqueous extracts to 12.5 ml. with water and filter. Mix 5 ml. of the filtrate with 1 ml. of 10% sodium cyanide solution.

Mix another 5-ml. portion of the filtrate with 1 ml. of 12.5% potassium dihydrogen phosphate solution. Read both solutions at 582 $m\mu$ and take the difference in readings.³⁶⁶

Alternatively, adjust the pH of a 30-ml. extract of the sample extract to pH 4.0-4.5. Add 1 ml. of 10% potassium cyanide solution. Extract with four 10-ml. portions of a 1:6 phenol-chloroform mixture. Add an excess of ether to the combined extracts, using a minimum of 80 ml. of ether. Shake with 2-ml. portions of water adjusted to pH 2.5 with hydrochloric acid until all color is extracted, usually 4-5 extractions. Adjust the volume of the extracts to 10 ml. Treat a 1-ml. aliquot with 1.4 ml. of hydrochloric acid adjusted to pH 2.5. Add 0.1 ml. of 10% potassium cyanide solution. Read at 550 $m\mu$ against a blank containing a 1-ml. aliquot of the sample and 1-ml. of hydrochloric acid at pH 2.5.³⁶⁷

By nitroso-R-salt and nitric acid. Dissolve the sample in 10 ml. of a 1:1 mixture of citrate buffer at pH 4 and water. For precise determinations, add a 0.5 ml. solution of 0.5 microgram of ⁶⁰ Cobalt-labelled vitamin B₁₂ per ml. to compute losses. Treat 100-150 mesh Amberlite CG-50 with sodium hydroxide solution and wash with water. Pass the solution through a 7-cm. column of the treated resin. Wash the column with 15 ml. of 1:110 hydrochloric acid and elute the vitamin with 10-12 ml. of 3:2 dioxan-1:47 hydrochloric acid. To the eluate, add 1 ml. of 2% sodium chloride solution and 1 ml. of concentrated nitric acid. Evaporate to dryness. Dissolve the residue in 70% perchloric acid and evaporate to dryness. Redissolve the residue in 70% perchloric acid. Again, evaporate to dryness and cool. Dissolve the residue in 3 ml. of acetate buffer at pH 6 and add 1 ml. of 0.05% nitroso-R-salt solution. Heat for 1 minute at 100° and add 1 ml. of concentrated nitric acid. Heat for 1 minute at 100°, cool, and dilute to 5 ml. Read at 520 $m\mu$ against a reagent blank.

As hydrocyanic acid. Macro. Add a 5-ml. sample containing approximately 10 mg. cyanocobalamin and 1 ml. of phosphoric acid to a 100-ml. flask. Suspend 1 ml. of a solution containing 0.25% of lithium carbonate and 0.50% of picric acid in the flask above the solution. Stopper, and heat at 50° in a thermostat illuminated by two 400-W mercury vapor lamps for 3.5 hours. Dilute the contents of the beaker to 5 ml. with pyridine and read at 506 $m\mu$.

³⁶⁶ I. DeCarneri, *Il Farmaco Ed. sci.* **10**, 31-6 (1955).

³⁶⁷ F. W. Van Klaveren, D. Banerji, P. C. Shrivastava and S. A. Patei, *Indian J. Pharm.* **16**, 36-41 (1954).

*Micro.*³⁶⁸ To 0.5-ml. of aqueous sample containing about 1 microgram of vitamin B₁₂, add 1 ml. of 15% tartaric acid solution. Place in a micro-diffusion apparatus and irradiate with ultraviolet light, thus distilling the liberated hydrocyanic acid isothermally into 1 ml. of 0.4% sodium hydroxide solution over a period of 6 hours. Chill the distillate to 0° and add 0.2 ml. of a 1:3 mixture of 0.1% chloramine-T solution and 13.8% monopotassium phosphate solution. Add 0.8 ml. of pyridine containing 0.1% of 4,4'-bis(3-methyl-1-phenyl-2-pyrazolin-5-one) and 0.5% of 3-methyl-1-phenyl-2-pyrazolin-5-one. After 90 minutes, read at 630 m μ . Correct for free cyanide radical in the sample by running a duplicate determination in the dark.

As cobalt by 1-(2-pyrridylazo)-2-naphthol. Injection preparations. To a cobalamin sample containing 0.05 mg. of cobalt, add 2 drops of concentrated sulfuric acid and heat to boiling. If the solution turns yellow, add 1 drop of hydrogen peroxide and heat for 3 minutes. Neutralize the colorless solution and adjust the pH to 5-6. Add 0.3 ml. of 1% 1-(2-pyrridylazo)-2-naphthol solution in 95% ethanol. After 5 minutes, add 5 ml. of 95% ethanol and 1 ml. of 1:10 sulfuric acid. Dilute to 25 ml. and cool to room temperature. Read at 585 m μ against a reagent blank.

Direct reading with uridine 5'-triphosphate. Pharmaceutical preparations. Wash Dowex 1-X8 resin, 200-400 mesh, successively with water, aqueous acetone, acetone, light petroleum, petroleum, acetone, aqueous acetone, water, concentrated hydrochloric acid, 1:1 hydrochloric acid and water until free from hydrochloric acid. Stir the resin for 30 minutes in 40 ml. of 0.4% sodium hydroxide solution. Filter, and wash with water until the washings are neutral to phenolphthalein.

Add the resin to a 20-cm. \times 8-mm. tube to form a 12-mm. layer. Add 5 ml. of a sample containing about 0.5 mg. of vitamin B₁₂ and 10 mg. of uridine 5'-triphosphate. Elute vitamin B₁₂ by the dropwise addition of 40 ml. of water. Elute uridine 5'-triphosphate with 190 ml. of 1:110 hydrochloric acid.

Dilute the vitamin B₁₂ eluate to 50 ml. and the uridine 5'-triphosphate eluate to 200 ml. Read the vitamin B₁₂ solution at 361 m μ . Dilute 10 ml. of the uridine 5'-triphosphate solution to 50 ml. with phosphate buffer for pH 7 and read at 262 m μ against 10 ml. of 1:110 hydrochloric acid diluted to 50 ml. with buffer. Cobalamins are also determined by developing the cobalt with 1-(2-pyrridylazo)-2-naphthol.³⁶⁹

³⁶⁸ A. Sazerat, *Ann. Pharm. Franc.* **22**, 159-60 (1964).

³⁶⁹ H. Flaschka and A. A. Abd. El Raheem, *Chemist Analyst* **46**, 95-7 (1957).

VITAMIN B_{12b}, HYDROXOCOBALAMIN

Hydroxocobalamin may be isolated or converted to cyanocobalamin. In either case, it is read directly.³⁷⁰ It may also be converted to the dicyanocobalamin as for vitamin B₁₂. When converted to cyanocobalamin and separated by paper chromatography, it is extracted with 1:1 phenol-chloroform.³⁷¹

For determination in the presence of cyanocobalamin, read the absorption in 0.04% sodium hydroxide solution and in 1:100 hydrochloric acid solution.³⁷² Calculate from the difference and check it against the readings at 350 m μ and 361 m μ in 1:100 hydrochloric acid solutions.

Procedure—*Direct reading. Total cobalamins.* Prepare the solution in acetate buffer for pH 5.5 to contain 0.025-0.3 mg. per ml. Read at 351 m μ and calculate as hydroxocobalamin.

Individual cobalamins. Chromatograph 0.1-0.2 ml. of a solution containing about 5 mg. of hydroxocobalamin per ml. on paper. Use a 10-cm. band on Whatman 3MM paper 9-12 \times 46 cm. Develop for 18 hours by the descending technic, using butanol saturated with water to which is added 1% of acetic acid, then water to incipient turbidity. Extract the individual bands with a 0.1% solution of potassium cyanide at pH 6.6. Read at 550 m μ as cyanocobalamin.

As the dicyanide. Use the method for vitamin B₁₂ by treatment with cyanide to form the dicyano derivative, but read at 367 m μ .

6-AZAUACIL AND AZAURIDINE

6-Azaauracil and its riboside, azauridine, are developed in alkaline solution with sodium nitroprusside.³⁷³

Procedure—*Fermentation broths. 6-Azaauracil.* Filter the sample through a 3-mm. layer of kieselguhr. Dilute 0.5 ml. to 7.3 ml. with water. Add 0.5 ml. of 0.5% sodium nitroprusside solution and 0.5 ml. of 1%

³⁷⁰ C. Cardini, G. Cavina, E. Cingolani, A. Mariani and C. Vicari, *Farmaco Ed. Prat.* **17**, 583-92 (1962).

³⁷¹ J. L. Martin and W. H. C. Shaw, *Analyst* **88**, 292-6 (1963).

³⁷² J. Bayer, *Pharmazie* **19**, 602-5 (1964).

³⁷³ E. Svátek, A. Čapek, and M. Tadra, *Českosl. farm.* **12**, 385-8 (1963).

sodium hydroxide solution. Set aside for 15 minutes. As a buffer, dissolve 25 grams of sodium acetate in 100 ml. of water and add 20 ml. of glacial acetic acid. Add 0.2 ml. of this to a portion of the sample and read at 500 $m\mu$.

Azauridine. Neutralize the sample and proceed as for 6-azauracil to determine both.

BISACODYL, 4,4'-(2-PYRIDYLMETHYLENE) DIPHENOL DIACETATE

Bisacodyl is read at 263 $m\mu$ after extraction from the sample into methanol.³⁷⁴ Tablet bases do not interfere. For 10 mg. of bisacodyl, the standard deviation is 0.228 mg. Beer's law is followed for 0.01-0.08 mg. per ml.

Procedure—Tablets. Extract a powdered sample containing approximately 15 mg. of bisacodyl with 100 ml. of methanol. Dilute 10 ml. of the extract with methanol to 50 ml. and read at 263 $m\mu$.

Suppositories. Dissolve the sample containing about 10 mg. of bisacodyl in 50 ml. of methanol at 50°. Cool and filter. Discard the first 5 ml. of the filtrate. Dilute the next 5-ml. portion to 50 ml. with methanol and read at 263 $m\mu$.

AMPROLIUM, 1-(4-AMINO-2-*n*-PROPYL-5-PYRIMIDINYLMETHYL)-2-PICOLINIUM CHLORIDE HYDROCHLORIDE

An earlier method³⁷⁵ called for extraction with 2:1 methanol-water, chromatographing on aluminum oxide and development with a mixed reagent of 2,7-naphthelane diol, potassium ferriocyanide, potassium cyanide, and sodium hydroxide in methanol for reading at 530 $m\mu$. This fluorometric method cuts the time to less than half.³⁷⁶ The reaction used is analogous to that for conversion of thiamine to thiochrome. The fluorescence is linear for 1-20 micrograms. The so-called amprochrome is stable in the dark for an hour. Normal inside artificial light effects it slowly and direct sunlight destroys 84% of the activity in an hour. The thiamine in a normal poultry feed has no effect on the results.

³⁷⁴ A. Muszalska, Dissert. Pharm. Krakow 15, 439-443 (1963).

³⁷⁵ E. P. Schultz and C. R. Szalkowski, *Anal. Chem.* 33, 795-8 (1961); C. R. Szalkowski and E. P. Schultz, *J. Assoc. Official Agric. Chemists* 44, 5-12 (1961).

³⁷⁶ J. Kanora and C. R. Szalkowski, *J. Assoc. Off. Agr. Chem.* 47, 209-13 (1964).

Procedure—Poultry feed. Blend a 5-gram sample for 5 minutes. Weigh an amount equivalent to about 0.75 mg. of amprolium. Mix for 30 minutes with 100 ml. of 5% trichloroacetic acid, using a magnetic stirrer. Filter, collecting about 10 ml. of clear filtrate. Dilute 5 ml. of the acid extract to 50 ml. with water. From here on, treat 15 ml. of the diluted sample extract, a reagent blank, and a standard in the same way.

To the 15-ml. portion, add 5 ml. of 30% sodium hydroxide solution. Mix well and immediately add 0.5 ml. of 5% silver nitrate solution. Mix well and after 2 minutes add 3 ml. of 2% potassium ferricyanide solution. Mix, and during the next 3 minutes, overlay with 15 ml. of *n*-butyl alcohol. Thereafter, shake vigorously for 1 minute and centrifuge for 1 minute. Mix 10 ml. of the clear butanol layer with 1 ml. of absolute ethanol. Activate a portion at 400 $m\mu$ and read at 455 $m\mu$.

THIABENDAZOLE, 2-(4'-THIAZOLYL)-BENZIMIDAZOLE

Thiabendazole is completely extracted from swine feed with dilute hydrochloric acid. After adjusting the pH to 6-8 it is extracted from most of the possible interferences with chloroform. Thiabendazole after reextraction with dilute hydrochloric acid is reduced with zinc dust in the presence of *p*-phenylenediamine. Oxidation by ferric ammonium sulfate gives a blue complex, extractable by butanol for reading at 600 $m\mu$.⁸⁷⁷ Nithiazide, enheptin, and sulfathiazole interfere. There is no interference by amprolium, arsanilic acid, acetyl-(*p*-nitrophenyl)-sulfanilamide, ethopabate, nicarbazin, sulfaquinoxaline, glycarbylamide, 3-nitro-4-hydroxyphenylarsonic acid, bithional, reserpine, phenothiazine, piperazine, zoalene, 3,5-dinitrobenzamide, chlorothiazide, choline chloride, riboflavin, butylated hydroxytoluene, butylated hydroxyanisole, thiamine, niacin, cystine, cysteine, procaine penicillin, streptomycin, methionine, furazolidone and nitrofurazone.

Procedure—Swine feed. Blend a 100-gram sample to pass a No. 30 sieve, usually for about 3 minutes. Weigh a sample expected to contain about 0.4 mg. of thiabendazole. Gently reflux, using a magnetic stirrer, for 30 minutes with 100 ml. of 1:110 hydrochloric acid. Cool, and centrifuge for about 5 minutes.

Mix 15 ml. of the clear extract with 0.05 ml. of 0.1% methyl red in ethanol. Discharge the red color by dropwise addition of 2% sodium hydroxide solution. The pH should now be 6.2-8. Add 5 grams of sodium

⁸⁷⁷ C. R. Szalkowski, *J. Assn. Off. Agr. Chem.* **47**, 235-79 (1964).

chloride and 20 ml. of chloroform. Shake on a mechanical shaker for 5 minutes and centrifuge for an equal time. Discard the aqueous layer. Shake 15 ml. of the chloroform layer with 20 ml. of 1:110 hydrochloric acid for five minutes. Heat 15 ml. of the aqueous acid layer at 100° for 15-20 minutes. This must drive off all the dissolved chloroform. Cool, and dilute to 25 ml. with 1:110 hydrochloric acid. Mix 15 ml. of the acid solution of the test substance with 5 ml. of fresh 0.05% solution of *p*-phenylenediamine dihydrochloride. Add 150 ± 5 mg. of zinc dust, stopper tightly, and mix to suspend the zinc dust. Let stand tightly stoppered for 2 minutes.

As a ferric reagent, dissolve 20 grams of ferric ammonium sulfate dodecahydrate in 75 ml. of water, add 10 ml. of 1:35 sulfuric acid, and dilute to 100 ml. The absorbance, when developed without a sample, should be less than 0.4 at 600 $m\mu$. Add 5 ml. of the ferric reagent to the reduced sample with a rapid-delivery pipet, immediately stopper, and shake about 15 seconds. Let the zinc settle for 3 minutes and decant the colored solution through a plug of glass wool to remove all zinc particles. After 45 minutes or more, shake 20 ml. of the colored solution with 4 grams of anhydrous sodium sulfate and 5 ml. of *n*-butanol. Centrifuge, and read the butanol layer at 600 $m\mu$ against *n*-butanol. Correct for a reagent blank.

GLYODIN, 2-HEPTADECYL-2-IMIDAZOLINE ACETATE

Glyodin is determined by reaction with bromophenol blue and chloroform extraction.³⁷⁸

Sample—*Apples, peaches, or pears.* Fill a wide-mouth gallon jar with apples, with no slack. Weigh, and add 250 ml. of isopropanol. Screw the cap over cellophane to minimize leakage. Tumble for 10 minutes and filter through glass wool. Repeat the stripping with another 250 ml. of isopropanol. Wash the funnel with isopropanol and dilute to 500 ml.

Procedure—As bromophenol blue reagent, dissolve 50 mg. in a small amount of water plus 2 ml. of glacial acetic acid. Dilute to 500 ml. with water. Evaporate 25 ml. of the isopropanol solution at 100° with an air jet. Add exactly 1 ml. of glacial acetic acid dropwise to wet all the residue. Cover with a watch glass and heat at 100° with swirling until

³⁷⁸ A. Kleinman, *J. Assoc. Offic. Agr. Chemists* **43**, 698 (1960); *ibid.* **44**, 214-18 (1961); *ibid.* **46**, 238-241 (1963).

the residue disintegrates. Rinse down the sides with chloroform and wash the beaker 4 times with chloroform. Dilute to 50 ml. with chloroform, disregarding turbidity and slight color.

Mix 25 ml. of the sample in chloroform and 25 ml. of the Bromophenol blue reagent. Shake for 1 minute and let separate for 20 minutes. Filter the chloroform layer through glass wool and read at 415 $m\mu$.

BENZIMIDAZOLONE AND ITS DERIVATIVES

Oxidation of benzimidazolone with chromic acid produces an intense red color that rapidly changes to blue and then fades. If the oxidation is carried out in a dilute solution in the presence of ethanol, the color is less intense but is more stable.³⁷⁹ The ethanol destroys excess chromic acid and prevents oxidative destruction of the colored product. Beer's law is followed for 0.02-0.3 micromole per liter. *o*-Phenylenediamine and its *p*-isomer interfere. 1-Phenyl-3-acetylbenzimidazolone and 1-phenylbenzimidazolone form colors with the reagent. *o*-Phenylenemalonamide, diacetyl *o*-phenylenediamine, and 1,2-ureylenenaphthalene do not interfere.

Procedure—Dissolve the sample in dilute sodium hydroxide or 10% ethanol, depending on its solubility. Dilute a 0.5-ml. portion of the sample containing 0.01-0.15 mg. of benzimidazolone with water and ethanol to 1.6 ml., the proportion of water to ethanol depending on the derivative being analyzed (see Table 35). Add 1.2 ml. of 3:2 sulfuric acid and cool

TABLE 35. OXIDATION OF BENZIMIDAZOLONE DERIVATIVES

<i>Benzimidazolone derivative</i>	<i>Wave length of max. abs., $m\mu$</i>	<i>Ml. of ethanol</i>
1,3-Dimethyl	525	0.95
1-Methyl	505	0.95
5- <i>tert</i> -Butyl	505	0.4
Benzimidazolone	505	0.4
5-Chloro	515	0.1

to room temperature. Add 0.2 ml. of 1% potassium dichromate solution and mix immediately. After 4-10 minutes, read at the indicated wave length for maximum absorption.

³⁷⁹ Curt C. Porter, *Anal. Chem.* 30, 2063-4 (1958).

THIOQUINOX, QUINOXALINE-2,3-TRITHIOCARBONATE

Thioquinox is saponified by ammonium hydroxide in the presence of sodium sulfide and nickel ion to form a red chelate. This cannot be filtered on paper, but is adsorbed. Acid converts the complex to the colorless nickel chelate. This can be extracted with benzene, and then reextracted into ammonium hydroxide as the color body.³⁸⁰ Recovery is about 70%.

Procedure—Fruit. Cut a sample of 1-2 kg. of large fruit into quarters and mix well. Peel 500 grams of this sample and cut the peel into small pieces. Shake the peel for 30 minutes with 500 ml. of *n*-hexane. Filter the decanted solvent through anhydrous sodium sulfate. Shake the sample 15 minutes with 250 ml. of *n*-hexane and filter. Extract small fruits, such as berries, whole.

Take a portion of the combined extracts to yield 0.01-0.1 mg. of thioquinox and concentrate to 10 ml. Slurry 13 grams of alumina with *n*-hexane and form a 5-cm. column in a 15-mm. chromatographic tube. Apply the concentrated extract and follow it with 60 ml. of *n*-hexane added in several portions, passing through at 2-3 ml. per minute. This is a clean-up step.

Evaporate the effluent to dryness *in vacuo*. Take up the residue in 2 ml. of methanol and add 0.1 ml. of 1% sodium sulfide solution. Mix and add 5 ml. of 1:1 ammonium hydroxide. Mix, and add 2 ml. of 10% nickel chloride solution. Mix, and add 3 ml. of 1:1 ammonium hydroxide. Let stand for 30 minutes to develop the red color. Normally, the solution is turbid with substances not removed in the clean-up step.

Acidify the solution with concentrated hydrochloric acid. It is now green. Extract the colorless nickel chelate with 20 ml. of benzene. Then shake the benzene extract with 2 ml. of 1:1 ammonium hydroxide to reform the red complex. Repeat this extraction with 2 ml. portions so long as the ammonia layer turns red. Dilute to a known volume and read at 530 $m\mu$ against 1:1 ammonium hydroxide.

DEOXYCYTIDINE

Deoxycytidine after chromatographic separation is developed by cysteine in strong sulfuric acid solution.³⁸¹

³⁸⁰ H. Tietz, M. F. Osman, H. Frehse and H. Niessen, *Pflanz-Nachrichten "Bayer"* 15, 166-71 (1962-3).

³⁸¹ O. Ya. Tereshchenko, *Lab. Delo* 10, 292-6 (1964).

Procedure—*Rat urine*. Acidify to pH 3 and cool to near zero. Filter and dilute with 1.5 volumes of water. Pass this through a column of KU-2 resin in the hydrogen form, over a period of 90 minutes, using 1 ml. of resin per 1.5 ml. of sample. Wash the column with water. Elute deoxycytidine with a volume of 1:14 ammonium hydroxide equal to the diluted sample above. Evaporate the eluate *in vacuo* at under 70°. Extract the dry residue with 1 ml. of methanol for each 2 ml. of original sample. Filter, and evaporate the filtrate *in vacuo* at under 50°. Take up the residue in 2 ml. of water and filter. Mix 0.5 ml. of this filtrate with 0.05 ml. of 3% cysteine hydrochloride solution and 5 ml. of 75% sulfuric acid. Read at 490 m μ after 20 hours.

ETHOQUIN, SANTOQUIN, 7-CHLORO-4-(4-DIETHYLAMINO-1-METHYL-BUTYLAMINE)-3-METHYLQUINOLINE

Ethoquine in petroleum ether is read fluorometrically.³⁸²

Procedure—*Feed*. Slurry a finely ground 10-gram sample with 50 ml. of methanol. After 10 minutes, decant through glass wool. Reextract with 50 ml. and 50 ml. of methanol. Dilute to 250 ml. Mix a 25-ml. aliquot with 100 ml. of water. Shake for 1 minute with 50 ml. of petroleum ether. If troublesome emulsions form, add about 0.1 gram of salt. Reextract with 25 ml. and 25 ml. of petroleum ether. Shake the combined extracts with 50 ml. of water and discard the washings. Dilute the petroleum ether to 100 ml. and read fluorometrically against quinine sulfate as 100.

GLUTETHIMIDE, 2-ETHYL-2-PHENYLGLUTARIMIDE, AND
4-ETHYL-4-PHENYLGLUTAMIC ACID

Glutethimide and 4-ethyl-4-phenylglutamic acid are separated by chromatography and read in the ultraviolet.³⁸³ For 2-ethyl-2-phenylglutarimide, glutethimide, in blood, plasma, or urine, extract with chloroform, remove interferences, and read at 235 m μ .³⁸⁴ Glutethimide is also

³⁸² Richard S. Gordon, Robert A. Conklin and Lawrence J. Machlin, *J. Assoc. Off. Agr. Chemists* **47**, 512-6 (1964).

³⁸³ E. Smith, *J. Pharm. Sci.* **53**, 942-4 (1964).

³⁸⁴ L. R. Goldbaum, M. A. Williams and T. Koppányi, *Anal. Chem.* **32**, 81-4 (1960).

reacted with formaldehyde and concentrated sulfuric acid to give a fluorescent compound.³⁸⁵

Procedure—Prepare a chromatographic column of 2 grams of Celite 545 impregnated with 2 ml. of 1:16 sulfuric acid above a column of 2 grams of Celite 545 impregnated with 1 ml. of 8.4% sodium bicarbonate solution. Wash the columns with 50 ml. of ether saturated with water. Then wash with 25 ml. of chloroform saturated with water. Suspend a sample of ground tablets containing about 250 mg. of glutethimide in 25 ml. of chloroform saturated with water. Add 10 ml. to the upper column and follow with 45 ml. and 45 ml. of chloroform similarly saturated. The entire chloroform goes through both columns. Discard the first 25 ml. of eluate but collect the next 90 ml. Dilute it to 100 ml. and read glutethimide at 257 m μ .

For 4-ethyl-4-glutaramic acid, a degradation product of glutarethimide, elute the lower column with 1% acetic acid saturated with chloroform. Read at 257 m μ .

OXYPHENISATIN, 3,3-bis(*p*-HYDROXYPHENYL) 2-INDOLINONE AND ITS DIACETATE

The test substances in alkaline ethanolic solutions are extracted with ether to remove interferences. Thereafter, the solution is acidified. If the diacetate is to be isolated, it is extracted with chloroform and cleaned up on a Celite column. The oxyphenisatin with or without diacetate is then similarly extracted with ether. Each, or the mixture, in ethanol gives a purple color with silver nitrate in mildly alkaline solution.³⁸⁶ The method conforms to Beer's law at 0.001-0.015 mg. per ml. Powders containing excipients are preextracted with 95% ethanol. A reaction with potassium ferrieyanide in alkaline solution gives a color for reading at 558 m μ .³⁸⁷

Sample—*Tablets*. Finely powder not less than 20 tablets. Weigh a sample equivalent to about 5 mg. of phenisatin or the diacetate and dissolve in 30 ml. of water by warming. Cool, and dilute to 100 ml. with 95% ethanol.

³⁸⁵ R. P. Haycock, P. B. Sheth and W. J. Mader, *J. Am. Pharm. Assoc., Sci. Ed.* **49**, 673-7 (1960).

³⁸⁶ Antoine Major, Jr., *J. Assoc. Official Agr. Chemists* **47**, 688-92 (1964).

³⁸⁷ Sixten Ljungberg, *Farm. Rev.* **49**, 241-51 (1950); M. Kahane and O. Sackur, *Ann. pharm. franc.* **11**, 103-9 (1953); Gunnar Hansen, *Arch. Pharm. Chemi* **66**, 1035-45 (1959).

Powders. Extract a portion equivalent to about 5 mg. of phenisatin or the diacetate in a Soxhlet apparatus with 95% ethanol for at least 5 hours. Concentrate the extract to about 50 ml. and dilute to 100 ml. with 95% ethanol.

Procedure—Place 0.5 gram of dry Celite 545 on a glass wool pad in the bottom of a chromatographic column. Mix 2 grams of the Celite with 2 ml. of 10.6% sodium carbonate solution and add it to the column. Press down firmly and cover with a pad of glass wool.

Transfer 10-ml. of the assay solution equivalent to about 0.5 mg. of oxyphenisatin or the diacetate to a separatory funnel. Add sufficient 0.4% sodium hydroxide solution and 95% ethanol so that the sample is in a 40% solution in 95% ethanol. Extract with 20 ml. of ether and transfer the aqueous layer to another funnel. Extract the ether layer with 15 ml. of 0.4% sodium hydroxide solution and 10 ml. of 95% ethanol. Add this extract to the second funnel and discard the ether. Add 50 ml. of water to the combined extracts and acidify with 1:1 hydrochloric acid.

To isolate oxyphenisatin diacetate, extract the acid solution with ten successive portions of chloroform. Percolate each successive extract through the chromatographic column. Evaporate the combined chloroform extracts to dryness and take up in an appropriate volume of 95% ethanol.

Extract the phenisatin with five successive 20 ml. portions of ether. In each case, pass the ether extract through the chromatographic column before adding the next extract. Evaporate the total ether percolate to dryness, take up in 95% ethanol, and dilute to 50 ml. with that solvent.

To 10 ml. of the ethanolic sample containing oxyphenisatin or the diacetate or both, add 4 drops of 1.8% silver nitrate solution and 15 ml. of 0.04% sodium hydroxide solution. During 90 minutes, the solution becomes yellow, then violet. Add a drop of concentrated ammonium hydroxide to clarify the solution and read at 565 $m\mu$ against a reagent blank.

THALIDOMIDE, 3-PHTHALIMIDOGlutARIMIDE

In 1:110 hydrochloric acid, the ultraviolet absorption spectrum of thalidomide shows maxima at 220 $m\mu$ and 299.5 $m\mu$. In absolute ethanol, the maximum is at 218 $m\mu$. When treated with alkali, the extinction decreases. This decrease is proportional to the concentration of thalidomide and is the basis for determination.³⁸⁸ The procedure is applicable to 0.001-

³⁸⁸ J. N. Green and B. C. Benson, *J. Pharm. Pharmacol.* **13**, 117T-121T (1961); R. Beckmann and H. H. Kampf, *Arzneimittel-Forsch.* **11**, 45-7 (1961).

0.005 mg. of thalidomide per ml. with standard error of $\pm 7.5\%$. When made alkaline, the 220 $m\mu$ band disappears and does not reappear on acidification. Basic impurities are removed by washing with dilute acid.

Treatment of thalidomide with alkaline hydroxylamine followed by ferric perchlorate gives the ferric-hydroxamic acid complex, which is read at 500 $m\mu$.³⁸⁹ An alternative is the use of ferrous sulfate.³⁹⁰

Procedure—*In the ultraviolet. Blood, plasma or urine.* Extract a 5-10 ml. sample with 50 ml. of chloroform by shaking vigorously for 5 minutes. Filter the extract and wash once by shaking with 5-10 ml. of 1:110 hydrochloric acid to remove basic impurities. Discard the acid wash and refilter the chloroform. Evaporate 40 ml. of clear filtrate to dryness in a vacuum desiccator.

Dissolve the dry residue in 1:110 hydrochloric acid at 60-65° and filter through acid-washed filter paper. Dilute to 8 ml. with acid. Read 3 ml. at 220 $m\mu$ against 1:110 hydrochloric acid. To both the sample and acid blank, add 0.5 ml. of 4% sodium hydroxide solution. Stir and read after 15 minutes at 220 $m\mu$. Repeat this procedure on a second 3-ml. aliquot of the sample and use the mean of the two values. Carry out the procedure on a blood sample containing no thalidomide. Calculate the extinction due to thalidomide as the difference between the mean value of the extinction of the sample and the extinction of the blood without thalidomide.

As the ferric hydroxamic acid complex. To 2 ml. of a solution of thalidomide, add 2 ml. of 14% hydroxylamine hydrochloride solution and 2 ml. of 14% sodium hydroxide solution. Keep below 5° for 1 hour. Add 2 ml. of 1:2 hydrochloric acid and 2 ml. of 35% ferrous ammonium sulfate solution. Dilute to 20 ml. with water and read the brown-purple color at 530 $m\mu$.

³⁸⁹ Tsukinaka Yamana and Takekazu Sato, *Arch. Preact. Pharm. Japan*, 22, 189-90 (1962).

³⁹⁰ Tsukinaka Yamana, Hiroshi Koike, Tie Murata and Kikui Murata, *Yakuzaigaku* 21, 32 (1961).

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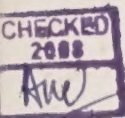
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